USGS
science for a changing world

H4IIE bioassay-derived 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents (TCDD-EQ) in fish collected in 2000 from large estuaries along the western coast of the United States.

Biomonitoring Environmental Status and Trends (BEST) and Environmental Monitoring and Assessment Program (EMAP) joint monitoring effort.

Biochemistry & Physiology Branch Final Laboratory Report FY 2004 – 30 – 01

6 October 2003

Prepared for: Tim Bartish
U.S. Geological Survey
Biomonitoring of Environmental
Status and Trends Program
2150 Centre Avenue, Bldg C
Ft. Collins, CO 80526-8118

Prepared by: Diane K. Nicks
Diane K. Nicks
Biologist, Biochemistry and Physiology Branch

Donald E. Tillitt
Donald E. Tillitt
Branch Chief, Biochemistry and Physiology Branch

U.S. Geological Survey
Columbia Environmental Research Center
4200 E. New Haven Road
Columbia, Missouri 65201

Citation

Nicks, D. K. and D. E. Tillitt, H4IIE bioassay-derived 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents (TCDD-EQ) in fish collected in 2000 from large estuaries along the western coast of the United States. USGS, Columbia Environmental Research Center, Biochemistry & Physiology Branch, Final laboratory report FY 2003 - 30 – 01, 2003, 16 p. (9 tables, 8 figures)

H4IIE bioassay-derived 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents (TCDD-EQ) in fish collected in 2000 from large estuaries along the western coast of the United States.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

The U.S. Fish and Wildlife Service initiated the Biomonitoring Environmental Status and Trends (BEST) Program in 1991 as a revision and expansion of the National Contaminants Biomonitoring Program (NCBP). NCBP was the Department of the Interior's program that was responsible for monitoring and documentation of temporal and geographical trends in the concentrations of persistent pesticides and industrial chemicals found throughout the nation. The BEST Program has an expanded scope, which includes measures of fish health. The H4IIE bioassay is one of the screening measures employed by the BEST Program to assess and characterize exposure to planar halogenated hydrocarbons (PHHs). The H4IIE bioassay is a semi-quantitative assay that measures the overall toxic potency of PHHs in the extracts of fish. PHHs consist largely of polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs). The information provided by the H4IIE bioassay complements the ecological metrics used in the Environmental Protection Agency's Environmental Monitoring and Assessment Program (EMAP). Thus, the H4IIE bioassay was conducted on fish portions that were collected as part of the western EMAP small estuaries assessment.

The BEST/EMAP collaboration envisioned a three-year joint effort to evaluate the ecological health of both small and large estuarine environments of California, Oregon and Washington, as well as the health of marine ecosystems associated with Alaska and Hawaii. The first phase of the study focused on the small estuaries of the western United States. The second, focused on numerous sites in three large estuaries, Puget Sound and the Strait of Georgia, the lower Columbia River and San Francisco Bay. To this end, predominantly benthic dwelling/feeding fish were collected, species tissues were composited, prepared, extracted and their extracts biologically evaluated by means of the H4IIE assay for the presence of contaminants.

The dioxin-like toxic potency of chemicals found in fish collected in these western estuaries is based on the ability of the extracts of those fish to increase 7-ethoxyresorufin-O-deethylase (EROD) activity in the H4IIE rat hepatoma cell line. The results of the induction caused by the extracts are evaluated relative to that of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The resultant TCDD equivalents (TCDD-EQs) are a measure of the exposure that the fish have received to this class of compounds.

OBJECTIVE

1) To determine H4IIE EROD bioassay-derived 2,3,7,8-tetrachloro-*p*-dioxin equivalents (TCDD-EQ) in extracts of fish collected at selected sites in Puget Sound and the Strait of Georgia, the lower Columbia River and San Francisco Bay.

MATERIALS AND METHODS

Sample History:

Benthic dwelling fish communities in the estuary waters of Puget Sound and the Strait of Georgia, Washington, the Lower Columbia River, Oregon and San Francisco Bay, California were sampled by trawl and seine during June-September of 2000. Sampling locations and sample identification are summarized for Washington (Table 1), Oregon (Table 2) and California (Table 3). Samples were stored frozen until homogenized. Sample fish were stored at -20^{o} C for up to 4-5 months before shipping to the Columbia Environmental Research Center (CERC), Columbia, MO. Ground fish composites were packed into pesticide free, amber I-Chem® jars and shipped to CERC via Federal Express Overnight Shipping. Samples arrived in Coleman Coolers packed in Blue Ice and wrapped in plastic bubble wrap. Sample transmittal and chain of custody forms accompanied samples during transmission. Upon receipt at CERC, samples were unpacked, logged in by Jesse Arms (CERC) and stored at -15^{o} C until processed for extraction. Forty-four composite fish samples from San Francisco Bay, California arrived on 2/14/2001. Fifty-seven samples arrived from the lower Columbia River, Oregon on 2/28/2001. Sixty-one composite fish samples were received from Puget Sound, Washington on 4/24/2001.

Analytical Sample Preparation Methods Summary:

All samples were assigned individual database identification numbers. Quality control (QC) samples (matrix blanks, procedural blanks, and positive control materials) were prepared concurrently with the test samples. Positive control material was derived from samples of CERC's standard positive control matrix, common carp (*Cyprinus carpio*) tissue, collected from Saginaw Bay, Michigan, December 1988. Matrix blank material was derived from clean bluegill (*Lepomis macrochirus*) raised in CERC's holding pond. Samples were defrosted at room temperature and 20 g aliquots removed and combined with a, four-fold excess by weight, of anhydrous sodium sulfate. After dehydrating overnight, the mixture was homogenized in a blender, packed into an extraction column, and extracted with methylene chloride (CH_2Cl_2). The resultant extract was concentrated via rotary evaporation. The concentrated extract was then subjected to reactive clean up per CERC SOP P.186 (Appendix 1). The samples were then prepared for the H4IIE bioassay by concentrating to near dryness followed by transfer to 300 µL dosing vials through a series of three rinses, reconstitution and subsequent concentration cycles. The samples were reconstituted in a final volume of 150 µL in iso-octane and stored in capped conical dosing vials until used for the bioassay.

H4IIE Bioassay Method:

The H4IIE bioassay procedure was a modification of that reported by Tillitt et al. (1991). These modifications miniaturize and expedite the assay procedure, by allowing for sample processing in 96 well microtiter plates, reported in CERC SOP C5.194 (Appendix 1). The H4IIE rat hepatoma cell line (American Type Culture Collection, ATCC) was maintained using standard sterile tissue culture techniques. Cells were cultured in

Dulbelcco's Modified Eagle's Medium (D-MEM) at 37°C, 5% CO_2 (ambient chamber concentration). Cell cultures and exposures occurred in a humidified, temperature and carbon dioxide level-controlled incubator (Forma Scientific, Marietta, Ohio). Microtiter plates were seeded by pipetting 300 µL of a media/cell suspension (approximately 23,000 cells/mL) into each well. Post seeding, cells were allowed to proliferate for approximately 24 hours. The cell containing plates were then dosed and returned to the incubator for 72 hours to allow for maximal EROD induction. Upon completion of this induction period, the EROD reaction was measured for each well on each plate.

A standardized 2,3,7,8-tetrachlorodibenzo-*p*-dioxin solution was used to generate an analytical dose-response curve to which all other samples would be related. Generally, six standard dose-response curves were measured on each assay date. Dose-response curves were prepared as a set of 7 serial dilutions along with an iso-octane blank for each sample or standard. The TCDD standard, (10 pg TCDD/µL iso-octane), was diluted in a ratio of 1:2 (v/v) while each experimental sample was diluted 1:3 (v/v). Six basal curves, non-dosed cells, to which were assigned an artificial dose were, also, run on each assay date. These were used to calculate basal induction, limits of detection (LOD), and limits of quantitation (LOQ). The TCDD standard and basal curves were placed in varying plate positions and interspersed among sample curves. All experimental fluorometric data were collected with a Perkin-Elmer BioSystems Cytofluor 4000 instrument.

A resorufin standard curve (range, 0 to 320 pmol) was generated on each assay date using the following procedure. Eight standard solutions were prepared by making a 1:1 (v/v) serial dilution of a prepared 16 µM resorufin/phosphate buffered saline (PBS) working stock. This working stock was prepared by making a dilution of a 200 µM, resorufin/methanol, super stock. The concentration of the super stock was checked spectrophotometrically at 571 nm on each assay date. Six replicates of each resorufin standard were added to a plate and the average background corrected arbitrary fluorescence units (AFU's) were plotted against the nominal resorufin concentrations to produce the resorufin standard curve and linear regression equation.

The reaction was initiated in experimental plates and fluorescence resulting from resorufin formation in each well was monitored once a minute for 20 minutes. The background corrected AFU's for the experimental plates were compared to the corresponding linear fit of the eight point resorufin standard curve and AFU's were converted into pmol of resorufin formed. The resorufin content in each well was plotted against time to evaluate any deviations from linearity in the progressive formation of resorufin with time. A linear regression analysis was performed on each sample well to obtain the slope and estimate the rate of reaction (pmol/min). The reaction rate observed in each well was normalized according to the measured protein content, generating a value of specific activity in units of pmols resorufin formed/(min*mg) of protein. Reported results are the average of at least four replicate curves. The linear portions of the slopes derived from each of these curves were normalized to the average initial slope obtained for the TCDD standard curves, resulting in a measure of an equivalent dose of TCDD (TCDD-EQ) for each sample.

The protein content in each well was determined using a fluorescamine-based protein assay (Undenfriend et al. 1972; Bohlen et al. 1973; Lorenzen and Kennedy, 1993). The reaction was allowed to progress for 10 minutes then fluorescence measurements were made. A separate BSA standard curve (range, 0 to 120 μg) was generated for each assay day. Eight standard solutions were prepared by making a 1:1 (v/v) serial dilution of a prepared 6 mg/mL BSA stock. Six replicates of each BSA standard were added to a plate and the average background corrected arbitrary fluorescence units (AFU's) were plotted against the nominal BSA concentrations to produce the standard curve and linear regression equation. Fluorescence values were measured for each sample well, the background corrected AFU's were compared to the corresponding linear fit of the eight point BSA standard curve and AFU's were converted into mg of protein.

All EROD assay reagents were incubated for ten minutes at 37^{o} C prior to data collection. The correct sample identification and its associated microtiter plate well were recorded on data log sheets and stored with the laboratory notebook. All electronic files were stored on CD's with names and other pertinent information recorded in the laboratory notebook.

Excess of the tissue extracts was stored at room temperature in sealed conical vials with the volume marked.

Quality Assurance and Quality Control:

The objective of the quality assurance plan of this study was to ensure that the biochemical analyses were accurate and representative measures of the TCDD-EQs found in each composite sample generated from those collected in the field portion of this study. The general scheme included replication of assayed samples, comparison of calibration against known standards, proper maintenance and calibration of equipment, accurate sample tracking and chain of custody, proper documentation at all steps of sample processing and other considerations of Good Laboratory Practice (GLP). The specific aspects of the QA plan related to the H4IIE EROD assay are given below.

All experimental information was recorded in bound notebooks and copies maintained in a separate, secured area. Instrument printouts and computer-generated data tables were uniquely labeled and cross-referenced to the project notebook. The accuracy of all such data reductions was independently verified. Hard copies of computerized data files were maintained in a project notebook. Computer files were backed-up and archived on CD's. All equipment used in this study was routinely inspected and preventive maintenance performed. A logbook was kept for each instrument to document its use, performance and maintenance.

Replication and subsequent performance checks were performed at many stages of the H4IIE EROD assay procedure. A composite TCDD dose-response curve was generated from the average of 6 independent determinations for each composite sample. Ten percent (10%) of tissue extract samples were assayed in triplicate, as were all positive

control and some matrix blank samples. Eight-point resorufin and BSA (bovine serum albumin) standard curves were prepared at 6 replicates for each concentration, and analyzed concurrently with the TCDD standards and samples. Positive control fish tissue extracts were analyzed on each assay date along with the samples. The source and lot number of the BSA and resorufin were recorded in the laboratory notebook. Scatter plots for the resorufin (Figure 1) and BSA (Figure 2) standard curves have been included with this report. The BSA standard curve data is presented in two separate graphs to account for an equipment upgrade that took place while samples for this project were being assayed. A 400 nm excitation filter was added to the Cytofluor 4000 instrument. BSA standard curves assayed with the previous 360 nm filter (CERC SOP C5.194, Appendix 1) are displayed in one graph and those assayed with the 400 nm filter are displayed in the second graph. These scatter plots were prepared to demonstrate the consistency of the fluorescence response with concentration over the time course of sample evaluation. They were also used to facilitate data analysis. The slope and y-intercept values were recorded electronically.

The concentrations of the resorufin, ethoxyresorufin and NADPH reagents were checked on each assay date using a spectrophotometer and their actual concentrations determined based upon Beer's Law using known extinction coefficients for the different reagents. It was deemed acceptable if the actual concentration was within 10% of the nominal concentration.

Positive control and matrix blank tissue extracts were included along with the samples for H4IIE analysis on each assay date to assure that both the EROD enzyme assay and the reagents were behaving according to specifications. The positive control was prepared from CERC's reference material, common carp from Saginaw Bay, Michigan. Fourteen 10g aliquots were separately extracted and carried through reactive absorbent clean-up columns in accordance with CERC's SOP P.186 (Appendix 1). Positive control extracts were designated with their own unique tracking number. Matrix blank extracts were prepared from pond-raised bluegill in the same process as that used for the positive control and were assigned a unique tracking number that was matched with a positive control sample.

Data Analysis:

The standard curve data for both the resorufin and BSA standard curves were separately compiled and each plotted as a set, in order to verify the consistency and consequently the reliability of the sample data as a whole. In each case, a linear regression, 95% confidence interval, and prediction interval were plotted (Figures 1 and 2, respectively). The means and error limits (SD or standard deviation, and CV or coefficient of variation) for both the resorufin and BSA standard data are given below.

	slope		y intercept	
	mean	(SD, CV)	mean	(SD, CV)
Resorufin	107 AFUs/pmol	(19.8, 18.5%)	102.8 AFUs	(233, 227%)
BSA	58 AFUs/mg	(28, 48 %)	186 AFUs	(146, 78%)

The time courses for the production of resorufin (i.e. the EROD reaction rates) were evaluated graphically to ensure linearity of the response. Linearity of the reactions verifies that non-saturating substrate levels or enigmatic kinetics did not limit the reactions. In cases where the kinetic reaction did not yield a linear response over the 20-minute sampling time, the linear portion of the curve was used to calculate the rate of reaction.

The linear portion of a plot of EROD specific activity versus gram tissue equivalents per mg cellular protein was used to determine the EROD induction response for the H4IIE cells for a given sample. This measure of EROD induction was translated into an equivalent dose of 2,3,7,8-tetrachloro-*p*-dioxin, TCDD, by dividing the average induction response arising from treatment of the cells with sample by that response arising from treatment of cells with the standard, TCDD (Eq. 1). The resultant metric for potency estimates was TCDD-equivalents (TCDD-EQs) in the extract or tissue sample.

$$\text{TCDD-EQ (pg/g)} = [(\text{EROD/g-equivalent in extract})/(\text{EROD/pg TCDD})] \quad (1)$$

Positive control and matrix blank samples were included during each data collection and work-up phase of this study. Three dose-response curves were run and evaluated for the positive control sample on each assay date. A composite dose-response curve was developed for the positive control samples used on each assay date. These were generated from combining raw data of individual dose-response curves. The slope and standard error were calculated from these composite curves. The positive control samples assayed in this study were plotted as TCDD-EQ versus assay date. These are illustrated in combination with line plots indicating the lab mean positive control value, high control limit (mean + 2 X SD), and low control limit (mean – 2 X SD) (Figure 3). Data for matrix blank samples were prepared in the same manner and illustrated in a second plot (Figure 4).

The degree of EROD induction in reagent blanks and basal cells were determined in addition to the measurement of EROD induction for the TCDD standard and positive controls. The limits of detection (LOD) and quantitation (LOQ) for each assay date were calculated, as described by Keith et al. (1983). These parameters were calculated from the observed level of basal EROD activity measured in the H4IIE cells on a given day. The LOD was defined to be equal to the average basal activity plus 3 times the standard deviation of the mean (standard error) associated with that activity. The LOQ was defined to be equal to the average basal activity plus 10 times the standard deviation of the mean (standard error) associated with that activity. These measures were used to evaluate the sample data results and to determine whether they were detectable or measurable above that of the background. Based upon the basal level of EROD activity found in uninduced H4IIE cells, the criteria of LOD and LOQ could be used to judge the significance of the measured results obtained for the samples. Control charts that evaluate the run-to-run variation of LOD and LOQ are presented (Figures 5 and 6 respectively). In each case, the value for each assay date is plotted in combination with line plots indicating the lab

mean value, high confidence limit (mean + 2 X SD), and low confidence limit (mean – 2 X SD).

Replicates of sample data were treated as separate samples until TCDD-EQ's were calculated. These were then averaged for reporting in tables and are indicated. A sample was re-run if the coefficient of variation was over 25%, starting with the concentrated tissue extract in iso-octane. Exceptions were made in the cases of the blanks and basal measurements or when the measured values fell below or very close to the limit of quantitation.

The H4IIE data of individual samples or mean data for those sites from which were assayed two or more samples were presented graphically on maps (Figures 7 – 9). Site data with individual sample results that fell into more then one category were represented on the map by the multiple categories (circle, triangle, or square of two colors). No sites had samples in more than two categories.

RESULTS and DISCUSSION

Quality Assurance

The data generated for all resorufin standard curves were compiled and plotted as background-corrected fluorescence values versus resorufin content per well (Figure 1). The linear regression, 95% confidence interval, and prediction interval for all of the data were plotted in Sigma Plot. As seen in the previous table the y-intercept coefficient of variation (CV), was 227% while that for the slope was18.5 %. While the y-intercept accounts for the day-to-day background fluctuations in the instrument, the slope reveals the correlation between fluorescence and resorufin content. Since the associated error in the slope was low, a high degree of confidence may be placed in the reliability of the mathematical conversion between measured fluorescence and calculated resorufin content throughout the course of the study.

The data generated for all BSA standard protein curves were compiled and plotted in the same manner as the resorufin with background-corrected fluorescence values versus protein content per well (Figure 2). The y-intercept coefficient of variation (CV) was 78% while that for the slope was 48%.

Generally, the results for all samples fell within the 25% CV limit as set forth by the QA objectives. Those samples that exceeded the 25% CV maximum fell into the reagent blank or basal classes of sample measurements or were samples that had calculated TCDD-EQs on the order of LOQ or less.

In order to evaluate the reproducibility of the experimental method throughout the time course of the analysis, the TCDD-EQs determined for the positive control and matrix blank samples were plotted as stated above (Figure 3 and 4). All positive control and matrix blank samples except one (7 %) in each case fell within set confidence intervals of the lab average. This variation is expected in any biological system.

Examination of the LOD (Figure 4) and LOQ (Figure 5) control charts illustrates that all values fell within limits except those for 1/11/02. Here, all values were skewed high and as all data is related to the TCDD curves run on the same day, TCDD-EQ's were well within normal range (i.e. PC110101 & MB110101).

Hazards of TCDD-EQ in the Aquatic Ecosystem

The H4IIE bioassay responds to chemicals that bind to the aryl hydrocarbon receptor (Ah-R). The chemicals included in this class are polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polycyclic aromatic hydrocarbons (PAHs). The H4IIE bioassay integrates the overall potency of these chemicals. The response of the cells to the extract of the environmental sample (fish in this case) is calibrated against 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and the resultant potency is given as TCDD-equivalents (TCDD-EQs). The extraction procedures used for preparation of the samples in this monitoring effort were designed to remove PAHs during the extraction and clean-up steps. Subsequently, the potency estimates of dioxin-like activity in fish derived from these tests are representative of the amounts of PCBs, PCDDs, and PCDFs in the samples. Other compounds may have been present in these fish and could have elicited a response in the H4IIE cells, however; PCBs, PCDDs, and PCDFs (collectively referred to as planar halogenated hydrocarbons or PHHs) are commonly the predominant chemical contaminants found in environmental samples (Giesy et al. 1994). The results of the H4IIE bioassay are to be used as a screening tool for the overall potency of dioxin-like chemicals present in the fish samples. The results can be used to prioritize further analysis or monitoring efforts, and make simple predictions about the relative risks expected from this class of compounds.

Results of the H4IIE bioassay can be categorized based on concerns due to dioxin-like effects. The H4IIE bioassay is a screen for dioxin-like potency observed in extracts of biotic and even abiotic environmental samples. The results of the assay are an integration of the total of the dioxin-like chemicals present in the extracts. The amounts of individual chemicals present in the environmental samples are not delineated with this assay. Chemical-specific risk assessments require traditional analytical chemistry data on individual chemicals. However, the data from the H4IIE bioassay can be used to conduct hazard ranking of the sites in this monitoring program. The hazards of dioxin-like chemicals are generally observed in species at the top of the trophic chain (ie. fish-eating birds and mammals). There are numerous toxic endpoints associated with dioxin-like chemicals, but the endpoints which are the most sensitive and the endpoints which are the most ecologically relevant to assessment of fish and wildlife populations are the reproductive effects (Peterson et al. 1990). In particular, rates of embryo lethality and birth deformities are important for understanding the effects of these compounds on a population. The results of the H4IIE bioassay were categorized to reflect the relative hazard that might be expected from the dioxin potency measured in the fish samples. The categories are relative benchmarks for comparative purposes and should not be construed as definitive thresholds of toxicity.

The relative hazard categories for the H4IIE bioassay results were based upon toxicity of the most sensitive life stage to the effects of dioxins, the developing embryo or fetus and the expected potential for biomagnification. Dioxins and other PHHs are known to biomagnify from fish to fish-eating birds and fish-eating mammals. Toxicity reference values (TRVs) of TCDD in fish and wildlife range from 35 pg TCDD/g egg in fish based on lake trout early life stage (ELS) mortality (Walker et al. 1994), to 100 pg TCDD/g egg for avian embryo lethality taken from a feeding study with ring-necked pheasant (Nosek et al. 1993). Reproductive toxicity of TCDD in mink occurs at 60 pg TCDD/g of liver in the adult mink (Tillitt et al. 1996). Taken together with the degree of biomagnification expected, a hazard category may be developed. The results of the H4IIE bioassay represent all of the PHHs present in the sample. These chemicals have a range of bioaccumulation or biomagnification potentials and for this reason, a chemical-specific risk assessment is not possible. However, if the potency of TCDD-EQs as measured by the H4IIE bioassay were evaluated as if they were derived from TCDD alone, then relative hazard categories may be developed. It is known that other Ah-R agonists, such as PCBs, are ubiquitous in the environment. The biomagnification factors for these other chemicals that contribute to the whole mixture of dioxin-like chemicals are sometimes greater and sometimes less than that of TCDD. Uses of the TCDD values simply provide a reference point from which to make relative screening categories. The biomagnification factor of TCDD from forage fish into predatory fish is approximately 1.0 (Cook et al. 1993; Jones et al. 2001). The biomagnification factor of TCDD from fish into the eggs of a fish-eating bird is approximately 20 (Braune and Norstom, 1989) and the biomagnification factor of TCDD from fish into mink livers is approximately 11 (Tillitt et al. 1996). Hazard categories may be developed from simple division of the toxicity reference values by the biomagnification factors (ie. TRV/BMF). Fish health may be expected to be impaired when TCDD-EQs in fish are 35 pg/g (TRV/BMF = 35/1). Avian reproductive health may be expected to be impaired when TCDD-EQs in fish are 5 pg/g (TRV/BMF = 100/20), while wildlife reproductive health can be expected to be impaired when TCDD-EQs in fish are 5 pg/g (TRV/BMF = 60/11). The TRV estimated by Tillitt et at. (1996) for dietary concentrations of TCDD equivalents in fish to protect mink reproductive health was 4.4 pg TCDD-EQ/g of fish. Thus, based on the potential for reproductive impairment and these biomagnification factors, H4IIE bioassay values in fish greater than 5 pg TCDD-EQ/g may be hazardous to avian and mammalian wildlife which consume fish. Therefore, general hazard categories for the concentrations of TCDD-EQs in fish have been set as: 1) not expected to be hazardous (LOD to LOQ); 2) potentially hazardous (measured values < 5 pg TCDD-EQs/g); and 3) likely to be hazardous (≥ 5 pg TCDD-EQs/g). The H4IIE bioassay data from these analyses were presented graphically based on these categories (Fig. 7-9).

TCDD-EQs in Fish

Most extracts of fish collected from sites in Puget Sound, Hood Canal, and near the San Juan and Gulf Islands had dioxin-like potencies that were less than 5 pg TCDD-EQs/g. These values indicate no hazard or only a potential hazard to fish eating birds & wildlife (Tables 4 & 7, Figure 7). Extracts from four sites in this sampling area, 31, 33, 40, and

55, did result in TCDD-EQ's in the hazardous range for aquatic ecosystems (Tables 4 & 7, Figure 7).

Whole fish extracts from 70% (31/44) of the sites in the lower Columbia River exhibited measurable dioxin-like potencies, with 52% (23/44) in the measurable to 5 pg TCDD-EQs/g category and 18% (8/44) in the greater than or equal to 5 pg TCDD-EQs/g category. This indicates that these sites are potentially hazardous or likely to be hazardous to fish eating birds & wildlife. Only 30% of the sites in the Columbia River were below LOQ and therefore not likely to be hazardous (Tables 5 & 8, Figure 8).

Extracts from only three sites in San Francisco Bay exhibited mean dioxin-like potency below quantitation. All other sites yielded extracts for one or more species that had measurable potencies in the less than 5 pg TCDD-Eqs/g or greater than 5 pg TCDD-EQs/g. Twenty of the thirty-one sites sampled yielded extracts of potential hazard and twelve of the thirty-one were measured to be likely hazardous (Tables 6 & 9, Figure 9).

Summary and Conclusions

Three large west coast estuaries, the Puget Sound area, the lower Columbia River, and San Francisco Bay, were surveyed for dioxin-like contamination in fish. The relative potency of dioxin-like chemicals found in benthic fish was assessed with a bioassay screening system, the H4IIE rat hepatoma cell line. The H4IIE bioassay measures all of the chemicals in an organic extract of the fish that bind to the dioxin receptor and cause dioxin-like toxicity. The results of the H4IIE bioassay may be used to highlight areas or environments in which dioxins or dioxin-like chemicals may be of concern. The H4IIE bioassay can be used to indicate those environments which need further chemical analysis, from those environments that do not need further chemical characterization for this class of compounds. The H4IIE bioassay results were categorized into 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents (TCDD-EQs) that were thought to be 1) no to low hazard; 2) potentially hazardous; 3) likely to be hazardous.

The amount of dioxin-like potency in the fish from these large estuaries varied. Fish extracts from sites in Puget Sound, Hood Canal and near the San Juan & Gulf Islands exhibited low TCDD-EQs. Only extracts from four sites were evaluated as likely hazardous. Many of the Columbia River extract samples exhibited measurable TCDD-EQs with a number of potentially to likely hazardous samples collected in the Reed Island, Rainier and Astoria areas. H4IIE bioassay analysis of the extracts collected in San Francisco Bay resulted in measurable TCDD-EQs in most cases with only one site below LOQ.

LITERATURE

Bohlen, P., S. Stein, W. Dairman, and S. Undenfriend (1973) Fluorometric assay of proteins in the nanogram range. *Arch. Biochem. and Biophys.* **155**, 213-220.

Braune, B.M. and R.J. Norstrom. (1989) Dynamics of organochlorine compounds in herring gulls: III. Tissue distribution and bioaccumulation in Lake Ontario gulls. *Environ. Toxicol. Chem.* **8**:957-968.

Cook, P.M., Erickson,R.J., Spehar, R.L, Bradbury, S.P., and Ankley, G.T. (1993). Interim report on data and methods for assessment of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin risks to aquatic life and associated wildlife. U.S. Environmental Protection Agency, Office of Research and Development Report EPA/600/R-63/055, Washingto, DC.

Giesy, J.P., J.P. Ludwig, and D.E. Tillitt. 1994. Dioxins, Dibenzofurans, PCBs and Colonial Fish-Eating Water Birds. In: Dioxins and Health, A. Schecter, Ed. Plenum Press, New York, NY. pp. 249-307.

Jones, P.D., K. Kannan, J.L. Newsted, D.E. Tillitt, L.L. Williams, and J.P. Giesy. (2001) Accumulation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin by rainbow trout (*Onchorhynchus mykiss*) at environmentally relevant dietary concentrations. *Environ. Toxicol. Chem.* **20**(2):344-350.

Keith, L.H., W. Crummett, J. Deegan, R.A. Libby, J.K. Taylor, G. Wentler (1983) Principles of environmental analysis. *Anal Chem* **55**:2210-2218.

Nosek, J.A., Sullivan, J.R., Craven, S.R., Gendron-Fitzpatrick, A., and Peterson, R.E. (1993) Embryotoxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in the ring-necked pheasant. *Environ. Toxicol. Chem*. **12**:1215-1222.

Peterson, R.E., Theobald, H.M., and Kimmel, G.L. (1993). Developmental and reproductive toxicity of dioxins and related compounds: Cross-species comparisons. *Crit. Rev. Toxicol*. **23**:283-335.

Tillitt, D.E., J.P. Giesy, and G.T. Ankley (1991) Characterization of the H4IIE rat hepatoma cell bioassay as a tool for assessing toxic potency of planar halogenated hydrocarbons in environmental samples. *Envir. Sci. and Technol.* **25 (1)**, 87-92.

Tillitt, D.E., R.W. Gale, J.C. Meadows, J.L. Zajicek, P.H. Peterman, S.N. Heaton, P.D. Jones, S.J. Bursian, T.J. Kubiak, J.P. Giesy, and R.J. Aulerich. (1996) Dietary Exposure of Mink to Carp from Saginaw Bay. 3. Characterization of Dietary Exposure to Planar Halogenated Hydrocarbons, Dioxin-equivalents, and Biomagnification. *Environ. Sci. Technol.* **30**(1):283-291.

Undenfriend, S., S. Stein, P. Bohlen, W. Dairman, W. Leimgruber and M. Weigele (1972) Fluorescamine: A reagent for the assay of amino acids, peptides, and primary amines in the picomole range. *Science* **178**, 871-872.

Walker, M.K., Cook, P.M., Batterman, A.R., Butterworth, B.C., Berini, C., Libal, J.J., Hufnagle, L.C. and Peterson, R.E. (1994). Translocation of 2,3,7,8-

tetrachlorodibenzo-*p*-dioxin from adult lake trout (*Salvelinus namaycush*) to oocytes: effects on early life stage development and sac fry survival. *Can. J. Fish. Aquat. Sci.* **51**, 1410-1419.

Table 1. Geographic location of EMAP sampling sites in Puget Sound and the Strait of Georgia

Station	Field Station I.D.	Composite I.D.	Sampling Site	Latitude (°N)	Longitude (°W)	Fish Collection Date	Species
WA00-0001	7	9764-1	Boundary Bay, west	48° 59' 3.012"	122° 59' 35.988"	8/1/2000	Rock Sole
WA00-0001	7	9764-2	Boundary Bay, west	48° 59' 3.012"	122° 59' 35.988"	8/1/2000	Rock Sole
WA00-0001	7	9767	Boundary Bay, west	48° 59' 3.012"	122° 59' 35.988"	8/1/2000	Starry Flounder
WA00-0002	12	9756	Boundary Bay, south	48° 57' 16.992"	122° 56' 58.812"	8/2/2000	English Sole
WA00-0003	17	9744	Cherry Point	48° 48' 55.0087"	122° 43' 7.79"	8/2/2000	English Sole
WA00-0004	29	9760	Bellingham Bay	48° 44' 58.992"	122° 29' 24.612"	7/31/2000	English Sole
WA00-0004	29	9766-1	Bellingham Bay	48° 44' 58.992"	122° 29' 24.612"	7/31/2000	Starry Flounder
WA00-0004	29	9766-2	Bellingham Bay	48° 44' 58.992"	122° 29' 24.612"	7/31/2000	Starry Flounder
WA00-0005	38	9749	Samish Bay/ Bellingham	48° 37' 31.008"	122° 31' 32.988"	7/28/2000	English Sole
WA00-0007	50	9751	Fidalgo Bay, inner	48° 29' 53.988"	122° 35' 58,812"	7/27/2000	Starry Flounder
WA00-0008	51	9750	Fidalgo Bay, inner	48° 29' 12.012"	122° 35' 12.012"	7/27/2000	English Sole
WA00-0009	66	9753	Saratoga Passage, north	48° 14' 34.008"	122° 37' 19.812"	7/25/2000	English Sole
WA00-0010	69	9769	Oak Harbor	48° 16' 28.02"	122° 39' 6.588"	7/26/2000	English Sole
WA00-0011	71	9763	Penn Cove	48° 13' 28.92"	122° 42' 38.052"	7/26/2000	English Sole
WA00-0013	85	9726	Possession Sound	48° 2' 19.032"	122° 18' 58.788"	6/20/2000	English Sole
WA00-0014	90	9747	Everett Harbor, middle	47° 58' 55.92"	122° 13' 19.812"	7/25/2000	English Sole

Table 1. Geographic location of EMAP sampling sites in Puget Sound and the Strait of Georgia

Station	Field Station I.D.	Composite I.D.	Sampling Site	Latitude (°N)	Longitude (°W)	Fish Collection Date	Species
WA00-0017	116	9739	Useless/Oak Bay	47° 58' 53.508"	122° 30' 12.168"	8/17/2000	English Sole
WA00-0018	119	9729	Possession Sound	47° 54' 24.264"	122° 20' 12.408"	6/20/2000	English Sole
WA00-0019	126	9746	Port Madison	47° 43' 33.672"	122° 31' 49.728"	7/24/2000	English Sole
WA00-0020	145	9758	Liberty Bay, outer	47° 42' 52.92"	122° 37' 45.48"	7/24/2000	Starry Flounder
WA00-0021	179	9736	Elliott Bay, northeast	47° 37' 26.184"	122° 22' 26.688"	7/18/2000	English Sole
WA00-0022	200	9735	Duamish River - East Wa	47° 35' 4.704"	122° 20' 44.808"	7/18/2000	English Sole
WA00-0023	207	9740	Port Ludlow	47° 55' 28.092"	122° 40' 46.2"	8/21/2000	English Sole
WA00-0024	209	9727	Hood Canal (north)	47° 50' 27.708"	122° 38' 45.348"	8/21/2000	English Sole
WA00-0025	214	9728	Port Gamble Bay	47° 50' 10.644"	122° 34' 42.6"	8/21/2000	English Sole
WA00-0030	226	9738	Hood Canal (south)	47° 22' 40.764"	123° 7' 44.832"	8/22/2000	English Sole
WA00-0031	229	9768	Port of Shelton	47° 12' 34.992"	123° 4' 58.08"	7/21/2000	Shiner Perch
WA00-0032	237	9742-1	Budd Inlet	47° 7' 45.372"	122° 54' 49.572"	7/20/2000	Speckled Sanddab
WA00-0032	237	9742-2	Budd Inlet	47° 7' 45.372"	122° 54' 49.572"	7/20/2000	Speckled Sanddab
WA00-0033	243	9724-1	Port of Olympia	47° 3' 5.904"	122° 53' 45.168"	7/21/2000	Pacific Staghorn Sculpin
WA00-0033	243	9724-2	Port of Olympia	47° 3' 5.904"	122° 53' 45.168"	7/21/2000	Pacific Staghorn Sculpin
WA00-0033	243	9745-1	Port of Olympia	47° 3' 5.904"	122° 53' 45.168"	7/21/2000	Shiner Perch

Table 1. Geographic location of EMAP sampling sites in Puget Sound and the Strait of Georgia

Station	Field Station I.D.	Composite I.D.	Sampling Site	Latitude (°N)	Longitude (°W)	Fish Collection Date	Species
WA00-0033	243	9745-2	Port of Olympia	47° 3' 5.904"	122° 53' 45.168"	7/21/2000	Shiner Perch
WA00-0034	252	9741	Case Inlet	47° 16' 10.452"	122° 51' 3.6"	7/20/2000	Starry Flounder
WA00-0035	260	9706	East Anderson Island/	47° 8' 54.132"	122° 39' 31.788"	6/16/2000	Dover Sole
WA00-0036	268	9743	Hale Passage	47° 15' 16.668"	122° 35' 53.16"	7/19/2000	English Sole
WA00-0037	269	9759	Gig Harbor	47° 20' 16.404"	122° 35' 4.128"	7/19/2000	English Sole
WA00-0038	273	9707	Colvos Passage	47° 30' 38.484"	122° 29' 9.168"	6/15/2000	Rock Sole
WA00-0038	273	9703	Colvos Passage	47° 30' 38.484"	122° 29' 9.168"	6/15/2000	Dover Sole
WA00-0040	286	9714	S.E. Commencement Bay	47° 17' 5.532"	122° 28' 19.452"	6/16/2000	Dover Sole
WA00-0041	304	9748	Hylebos Waterway	47° 16' 43.14"	122° 23' 54.312"	7/19/2000	English Sole
WA00-0042	E1	9762	Puget Sound	48° 56' 14.028"	123° 44' 7.008"	8/6/2000	Rock Sole
WA00-0042	E1	9762	Puget Sound	48° 56' 14.028"	123° 44' 7.008"	8/6/2000	Rock Sole
WA00-0045	E4	9719	Puget Sound	48° 56' 13.488"	123° 12' 4.5"	6/24/2000	Dover Sole
WA00-0046	E5	9730	Puget Sound	48° 57' 19.98"	123° 0' 14.04"	8/1/2000	Rock Sole
WA00-0047	E6	9757	Puget Sound	48° 54' 5.148"	122° 55' 31.332"	8/3/2000	English Sole
WA00-0048	E7	9732	Puget Sound	48° 48' 17.928"	123° 23' 42.108"	8/3/2000	English Sole
WA00-0049	E8	9723	Puget Sound	48° 42' 42.768"	123° 31' 41.736"	8/3/2000	English Sole

Table 1. Geographic location of EMAP sampling sites in Puget Sound and the Strait of Georgia

Station	Field Station I.D.	Composite I.D.	Sampling Site	Latitude (°N)	Longitude (°W)	Fish Collection Date	Species
WA00-0050	E9	9733	Puget Sound	48° 49' 25.86"	122° 43' 48.972"	8/2/2000	English Sole
WA00-0054	E13	9722	Puget Sound	48° 34' 17.652"	123° 20' 7.476"	8/7/2000	Starry Flounder
WA00-0055	E14	9752	West Sound	48° 37' 31.728"	122° 57' 37.872"	8/10/2000	English Sole
WA00-0056	E15	9765	San Juan Channel	48° 36' 42.012"	123° 0' 4.68"	8/10/2000	Rock Sole
WA00-0058	E17	9770	East Sound	48° 36' 38.484"	122° 50' 15.18"	8/15/2000	English Sole
WA00-0059	E18	9761	San Juan Channel	48° 32' 40.308"	122° 58' 47.172"	6/22/2000	Pacific Tomcod
WA00-0066	E25	9731	Puget Sound	48° 18' 57.24"	122° 47' 59.712"	8/16/2000	English Sole
WA00-0068	E27	9734	Puget Sound	48° 7' 11.784"	122° 37' 24.024"	8/17/2000	English Sole
WA00-0069	E28	9708	Puget Sound	47° 57' 57.132"	122° 33' 14.58"	6/25/2000	Rock Sole
WA00-0069	E28	9725	Puget Sound	47° 57' 57.132"	122° 33' 14.58"	6/25/2000	English Sole
WA00-0070	E29	9718	Puget Sound	47° 51' 57.672"	122° 25' 10.056"	6/19/2000	Rock Sole
WA00-0071	E30	9709	Puget Sound	47° 35' 24.576"	122° 25' 42.06"	6/15/2000	Dover Sole

Table 2. Geographic location of EMAP sampling sites in the lower Columbia River

Station	Composite ID	Sampling Site	Latitude (°N)	Longitude (°W)	Fish Collection Date	Species
OR2000-0002	ODEQ002A	Columbia River, Upstream of Wallace Island	46° 10' 8.4"	123° 12' 57.6"	8/29/2000	Northern Pikeminnow
OR2000-0003	ODEQ003A	Columbia River, North of Crims Island	46° 11' 16.8"	123° 8' 27.6"	8/29/2000	Northern Pikeminnow
OR2000-0003	ODEQ003B	Columbia River, North of Crims Island	46° 11' 16.8"	123° 8' 27.6"	9/15/2000	Northern Pikeminnow
OR2000-0004	ODEQ004A	Columbia River, North of Wallace Island	46° 8' 31.2"	123° 16' 30.0"	9/15/2000	Starry Flounder
OR2000-0005	ODEQ005A	Columbia River, Barlow Pt.	46° 8' 45.6"	123° 2' 9.6"	10/2/2000	Northern Pikeminnow
OR2000-0005	ODEQ005B	Columbia River, Barlow Pt.	46° 8' 45.6"	123° 2' 9.6"	10/2/2000	Peamouth
OR2000-0007	ODEQ007A	Columbia River, NE of Slaughter's Dike	46° 6' 50.4"	122° 58' 40.8"	10/31/2000	White Sturgeon
OR2000-0007	ODEQ007B	Columbia River, NE of Slaughter's Dike	46° 6' 50.4"	122° 58' 40.8"	10/31/2000	Peamouth
OR2000-0009	ODEQ009B	Columbia River, East Ranier	46° 5' 20.4"	122° 55' 22.8"	11/7/2000	Peamouth
OR2000-0010	ODEQ010A	Columbia River, Prescott Beach	46° 3' 25.2"	122° 53' 13.0"	10/6/2000	White Sturgeon
OR2000-0011	ODEQ011A	Columbia River, North Kalama	46° 0' 57.6"	122° 51' 28.8"	9/26/2000	White Sturgeon
OR2000-0012	ODEQ012A	Columbia River, Deer Island Slough	45° 54' 43.2"	122° 48' 36.0"	10/30/2000	White Sturgeon
OR2000-0013	ODEQ013A	Columbia River, North St. Helens	45° 52' 37.2"	122° 47' 34.8"	10/4/2000	White Sturgeon
OR2000-0014	ODEQ014A	Columbia River, South St. Helens (North of Sand Island)	45° 51' 54.0"	122° 47' 16.8"	10/3/2000	Peamouth

Table 2. Geographic location of EMAP sampling sites in the lower Columbia River

Station	Composite ID	Sampling Site	Latitude (°N)	Longitude (°W)	Fish Collection Date	Species
OR2000-0015	ODEQ015A	Columbia River, Warrior Rock (North Sauvie Island)	45° 50' 45.6"	122° 47' 9.6"	10/30/2000	Northern Pikeminnow
OR2000-0016	ODEQ016D	Columbia River, Kelley Pt.	45° 39' 7.2"	122° 45' 46.8"	9/25/2000	White Sturgeon
OR2000-0017	ODEQ017A	Columbia River, Downstream End of Hayden Island	45° 38' 42.0"	122° 45' 3.6"	9/22/2000	Peamouth
OR2000-0019	ODEQ019A	Columbia River, North of West Lemon Island	45° 35' 49.2"	122° 34' 8.4"	9/26/2000	Peamouth
OR2000-0020	ODEQ020A	Columbia River, SW of Lemon Island (PDX Tower to South)	45° 35' 38.4"	122° 34' 55.2"	9/8/2000	White Sturgeon
OR2000-0020	ODEQ020B	Columbia River, SW of Lemon Island (PDX Tower to South)	45° 35' 38.4"	122° 34' 55.2"	9/26/2000	White Sturgeon
OR2000-0021	ODEQ021A	Columbia River, N. Channel-Mid Government Island	45° 35' 2.4"	122° 30' 7.2"	9/5/2000	Peamouth
OR2000-0021	ODEQ021B	Columbia River, N. Channel-Mid Government Island	45° 35' 2.4"	122° 30' 7.2"	9/26/2000	White Sturgeon
OR2000-0021	ODEQ021C	Columbia River, N. Channel-Mid Government Island	45° 35' 2.4"	122° 30' 7.2"	9/26/2000	Northern Pikeminnow
OR2000-0022	ODEQ022A	Columbia River, East of Sandy River Mouth (1)	45° 34' 4.8"	122° 21' 57.6"	10/13/2000	Peamouth
OR2000-0023	ODEQ023A	Columbia River, East of Sandy River Mouth (2)	45° 34' 8.4"	122° 21' 46.8"	9/5/2000	White Sturgeon
OR2000-0023	ODEQ023B	Columbia River, East of Sandy River Mouth (2)	45° 34' 8.4"	122° 21' 46.8"	9/5/2000	Northern Pikeminnow
OR2000-0024	ODEQ024A	Columbia River, Beacon Rock	45° 37' 22.8"	122° 1' 4.8"	9/1/2000	White Sturgeon

Table 2. Geographic location of EMAP sampling sites in the lower Columbia River

Station	Composite ID	Sampling Site	Latitude (°N)	Longitude (°W)	Fish Collection Date	Species
OR2000-0025	ODEQ025A	Columbia River, East of Franz Lake	45° 36' 18.0"	122° 3' 10.8"	9/1/2000	White Sturgeon
OR2000-0026	ODEQ026A	Columbia River, Norht of Reed Island	45° 33' 21.6"	122° 18' 0.0"	11/8/2000	White Sturgeon
OR2000-0027	ODEQ027A (composite of 0027 & 0030)	Columbia River, East of Phoca Rock (near boat ramp)	45° 34' 30.0"	122° 9' 54.0"	9/28/2000	White Sturgeon
OR2000-0027	ODEQ027B	Columbia River, East of Phoca Rock (near boat ramp)	45° 34' 30.0"	122° 9' 54.0"	9/28/2000	Peamouth
OR2000-0028	ODEQ028A	Columbia River, South of Reed Island	45° 32' 45.6"	122° 18' 54.0"	10/13/2000	Peamouth
OR2000-0028	ODEQ028B	Columbia River, South of Reed Island	45° 32' 45.6"	122° 18' 54.0"	10/13/2000	White Sturgeon
OR2000-0028	ODEQ028C	Columbia River, South of Reed Island	45° 32' 45.6"	122° 18' 54.0"	10/13/2000	Northern Pikeminnow
OR2000-0029	ODEQ029A	Columbia River, East of Reed Island	45° 33' 0.0"	122° 16' 15.6"	11/8/2000	Starry Flounder
OR2000-0029	ODEQ029B	Columbia River, East of Reed Island	45° 33' 0.0"	122° 16' 15.6"	11/8/2000	Peamouth
OR2000-0030	ODEQ030A	Columbia River, SW of Skamania I	45° 34' 51.6"	122° 8' 56.4"	9/1/2000	Peamouth
OR2000-0030	ODEQ030B	Columbia River, SW of Skamania I	45° 34' 51.6"	122° 8' 56.4"	9/28/2000	White Sturgeon
OR2000-0031	ODEQ031A	Columbia River Mouth: west of Jetty A	46° 16' 15.6"	124° 2' 42.0"	10/26/2000	Pacific Staghorn Sculpin

Table 2. Geographic location of EMAP sampling sites in the lower Columbia River

Station	Composite ID	Sampling Site	Latitude (°N)	Longitude (°W)	Fish Collection Date	Species
OR2000-0032	ODEQ032A	Columbia River, Sw of Sand Island Tower	46° 15' 32.4"	124° 1' 15.6"	10/26/2000	Pacific Staghorn Sculpin
OR2000-0033	ODEQ033A	Columbia River, East of Clatsop Spit	46° 13' 37.2"	123° 58' 40.8"	9/26/2000	Pacific Staghorn Sculpin
OR2000-0033	ODEQ033B	Columbia River, East of Clatsop Spit	46° 13' 37.2"	123° 58' 40.8"	9/26/2000	English Sole
OR2000-0034	ODEQ034A	Columbia River, "North Shore" (E. of N. Astoria Bridge)	46° 14' 45.6"	123° 51' 54.0"	10/25/2000	Pacific Staghorn Sculpin
OR2000-0035	ODEQ035A	Columbia River, West of Grays Pt.	46° 16' 58.8"	123° 47' 34.8"	10/24/2000	Pacific Staghorn Sculpin
OR2000-0036	ODEQ036A	Columbia River, Mid-river between Hammond, OR and Chinook Pt., WA	46° 13' 55.2"	123° 56' 20.4"	10/24/2000	Pacific Staghorn Sculpin
OR2000-0037	ODEQ037A	Columbia River, East of N. Astoria Bridge	46° 14' 31.2"	123° 51' 32.4"	10/25/2000	Pacific Staghorn Sculpin
OR2000-0038	ODEQ038A	Columbia River, Pt. Ellice (SW of North Astoria Gridge)	46° 14' 2.4"	123° 52' 48.0"	10/24/2000	Pacific Staghorn Sculpin
OR2000-0039	ODEQ039A	Columbia River, North of Taylor Sands	46° 14' 20.4"	123° 47' 24.0"	10/25/2000	Pacific Staghorn Sculpin
OR2000-0040	ODEQ040A	Columbia River, North of Rice Island	46° 16' 8.4"	123° 42' 46.8"	9/20/2000	White Sturgeon
OR2000-0041	ODEQ041A	Columbia River, Desdemona Sands (Downstream of Astoria Bridge)	46° 12' 18.0"	123° 52' 55.2"	8/22/2000	Pacific Staghorn Sculpin
OR2000-0042	ODEQ042A	Columbia River, West of Taylor Sands	46° 13' 19.2"	123° 47' 49.2"	10/25/2000	English Sole

Table 2. Geographic location of EMAP sampling sites in the lower Columbia River

Station	Composite ID	Sampling Site	Latitude (°N)	Longitude (°W)	Fish Collection Date	Species
OR2000-0043	ODEQ043A	Columbia River, SW of Rice Island	46° 14' 24.0"	123° 43' 55.2"	10/25/2000	Pacific Staghorn Sculpin
OR2000-0044	ODEQ044A	Columbia River, Altoona	46° 15' 50.4"	123° 39' 28.8"	9/20/2000	Pacific Staghorn Sculpin
OR2000-0045	ODEQ045B	Columbia River, East of Jim Crow Sands; West of Jim Crow Pt.	46° 15' 14.4"	123° 33' 43.2"	10/23/2000	Pacific Staghorn Sculpin
OR2000-0046	ODEQ046B	Columbia River, NW of Tongue Pt. Coast Guard Station	46° 12' 46.8"	123° 46' 51.6"	9/12/2000	Pacific Staghorn Sculpin
OR2000-0046	ODEQ046C	Columbia River, NW of Tongue Pt. Coast Guard Station	46° 12' 46.8"	123° 46' 51.6"	9/12/2000	Pacific Staghorn Sculpin
OR2000-0047	ODEQ047A	Columbia River, North of Green Marsh	46° 13' 19.2"	123° 39' 54.0"	10/25/2000	Pacific Staghorn Sculpin
OR2000-0048	ODEQ048A	Columbia River, North of Fitzpatrick	46° 16' 4.8"	123° 30' 7.2"	9/18/2000	Pacific Staghorn Sculpin

Table 3. Geographic location of EMAP sampling sites in San Francisco Bay, CA (Fish collection dates not provided.)

Station	Composite ID	Sampling Site	Latitude (°N)	Longitude (°W)	Species
CA00-0001	01A	San Francisco Bay, CA	37° 42' 55.1"	122° 22' 1.2"	Pacific Staghorn Sculpin
CA00-0002	02A	San Francisco Bay, CA	37° 47' 43.5"	122° 19' 15.6"	English Sole
CA00-0002	02B	San Francisco Bay, CA	37 ° 47' 43.5"	122 ° 19' 15.6"	Pacific Staghorn Sculpin
CA00-0003	03A	San Francisco Bay, CA	37 ° 48' 9.324"	122 ° 20' 42.0"	English Sole
CA00-0003	03B	San Francisco Bay, CA	37° 48' 9.324"	122 ° 20' 42.0"	Pacific Staghorn Sculpin
CA00-0004	04A	San Francisco Bay, CA	37 ° 45' 7.02"	122 ° 12' 54.0"	Starry Flounder
CA00-0006	06A	San Francisco Bay, CA	37 ° 37' 7.97"	122 ° 19' 48.0"	CA Halibut
CA00-0012	12A	San Francisco Bay, CA	37 ° 35' 40.92"	122 ° 10' 33.6"	CA Halibut
CA00-0014	14A	San Francisco Bay, CA	37 ° 27' 45.07"	122 ° 1' 22.8"	Pacific Staghorn Sculpin
CA00-0015	15A	San Francisco Bay, CA	37 ° 26' 59.172"	122 ° 1' 12.0"	Pacific Staghorn Sculpin
CA00-0016	16A	San Francisco Bay, CA	37 ° 52' 3.468"	122 ° 29' 42.0"	English Sole
CA00-0016	16B	San Francisco Bay, CA	37 ° 52' 3.468"	122 ° 29' 42.0"	Pacific Staghorn Sculpin
CA00-0017	17A	San Francisco Bay, CA	37° 52' 1.452"	122 ° 21' 43.2"	CA Halibut
CA00-0017	17B	San Francisco Bay, CA	37 ° 52' 1.452"	122 ° 21' 43.2"	Pacific Staghorn Sculpin
CA00-0019	19A	San Francisco Bay, CA	38 ° 5' 25.008"	122 ° 3' 28.8"	Pacific Staghorn Sculpin
CA00-0024	24A	San Francisco Bay, CA	38 ° 8' 32.64"	122 ° 16' 58.8"	Starry Flounder
CA00-0025	25A	San Francisco Bay, CA	38 ° 7' 43.104"	122 ° 16' 55.2"	Starry Flounder
CA00-0027	27A	San Francisco Bay, CA	38 ° 5' 48.696"	122 ° 21' 3.6"	Pacific Staghorn Sculpin
CA00-0029	29A	San Francisco Bay, CA	38 ° 2' 36.168"	122 ° 21' 32.4"	CA Halibut
CA00-0030	30A	San Francisco Bay, CA	38 ° 1' 43.284"	122 ° 19' 55.2"	Pacific Staghorn Sculpin
CA00-0030	30B	San Francisco Bay, CA	38 ° 1' 43.284"	122 ° 19' 55.2"	Starry Flounder
CA00-0031	31A	San Francisco Bay, CA	37 ° 57' 47.7"	122 ° 28' 12.0"	CA Halibut
CA00-0031	31B	San Francisco Bay, CA	37 ° 57' 47.7"	122 ° 28' 12.0"	Starry Flounder

Table 3. Geographic location of EMAP sampling sites in San Francisco Bay, CA (Fish collection dates not provided.)

Station	Composite ID	Sampling Site	Latitude (°N)	Longitude (°W)	Species
CA00-0032	32A	San Francisco Bay, CA	37 ° 54' 6.552"	122 ° 27' 50.4"	CA Halibut
CA00-0032	32B	San Francisco Bay, CA	37 ° 54' 6.552"	122 ° 27' 50.4"	Pacific Staghorn Sculpin
CA00-0033	33A	San Francisco Bay, CA	37 ° 56' 2.832"	122 ° 25' 4.8"	CA Halibut
CA00-0034	34A	San Francisco Bay, CA	37 ° 52' 48.252"	122 ° 22' 58.8"	CA Halibut
CA00-0034	34B	San Francisco Bay, CA	37 ° 52' 48.252"	122 ° 22' 58.8"	Pacific Staghorn Sculpin
CA00-0035	35A	San Francisco Bay, CA	37 ° 50' 1.176"	122 ° 21' 14.4"	CA Halibut
CA00-0035	35B	San Francisco Bay, CA	37 ° 50' 1.176"	122 ° 21' 14.4"	Pacific Staghorn Sculpin
CA00-0036	36A	San Francisco Bay, CA	37 ° 48' 42.84"	122 ° 20' 49.2"	English Sole
CA00-0036	36B	San Francisco Bay, CA	37 ° 48' 42.84"	122 ° 20' 49.2"	Pacific Staghorn Sculpin
CA00-0037	37A	San Francisco Bay, CA	37 ° 52' 40.8"	122 ° 28' 40.8"	CA Halibut
CA00-0037	37B	San Francisco Bay, CA	37 ° 52' 40.8"	122 ° 28' 40.8"	Pacific Staghorn Sculpin
CA00-0038	38A	San Francisco Bay, CA	38 ° 12' 11.232"	122 ° 34' 8.4"	Pacific Staghorn Sculpin
CA00-0042	42A	San Francisco Bay, CA	38 ° 8' 47.94"	122 ° 18' 50.4"	CA Halibut
CA00-0044	44A	San Francisco Bay, CA	37 ° 57' 59.76"	122 ° 28' 12.0"	CA Halibut
CA00-0044	44B	San Francisco Bay, CA	37 ° 57' 59.76"	122 ° 28' 12.0"	Pacific Staghorn Sculpin
CA00-0044	44C	San Francisco Bay, CA	37 ° 57' 59.76"	122 ° 28' 12.0"	Starry Flounder
CA00-0045	45A	San Francisco Bay, CA	37 ° 45' 57.132"	122 ° 13' 33.6"	CA Halibut
CA00-0046	46A	San Francisco Bay, CA	37 ° 30' 16.884"	122 ° 10' 37.2"	Starry Flounder
CA00-0048	48A	San Francisco Bay, CA	38 ° 1' 45.408"	122 ° 29' 27.6"	CA Halibut
CA00-0049	49A	San Francisco Bay, CA	37 ° 40' 29.136"	122 ° 22' 1.2"	CA Halibut
CA00-0050	50A	San Francisco Bay, CA	37 ° 41' 6.072"	122 ° 12' 21.6"	CA Halibut

Table 4. TCDD equivalents (pg/g) of EMAP samples taken in Puget Sound and the Straight of Georgia

Station	Composite ID	Sampling Site	Species	TCDD-EQ (pg/g)	SD	
WA00-0001	40-3764-1	Boundary Bay, west	Rock Sole	1.5	0.3	Mean of 3
WA00-0001	40-3767	Boundary Bay, west	Starry Flounder	2.7	0.4	
WA00-0001	40-3764-2	Boundary Bay, west	Rock Sole	1.6	0.3	
WA00-0002	40-3756	Boundary Bay, south	English Sole	<LOQ		
WA00-0003	40-3744	Cherry Point	English Sole	<LOQ		
WA00-0004	40-3760	Bellingham Bay	English Sole	3.4	0.4	
WA00-0004	40-3766-1	Bellingham Bay	Starry Flounder	<LOQ		
WA00-0004	40-3766-2	Bellingham Bay	Starry Flounder	4.1	0.7	
WA00-0005	40-3749	Samish Bay/ Bellingham	English Sole	1.5	0.2	
WA00-0007	40-3751	Fidalgo Bay, inner	Starry Flounder	<LOQ		
WA00-0008	40-3750	Fidalgo Bay, inner	English Sole	<LOQ		
WA00-0009	40-3753	Saratoga Passage, north	English Sole	<LOD		
WA00-0010	40-3769	Oak Harbor	English Sole	<LOD		
WA00-0011	40-3763	Penn Cove	English Sole	<LOQ		
WA00-0013	39-9726	Possession Sound	English Sole	1.2	0.3	
WA00-0014	40-3747	Everett Harbor, middle	English Sole	2.1	0.3	Mean of 3
WA00-0017	39-3739	Useless/Oak Bay	English Sole	2.7	0.5	
WA00-0018	39-9729	Possession Sound	English Sole	3.1	0.4	
WA00-0019	40-3746	Port Madison	English Sole	3.5	0.4	
WA00-0020	40-3758	Liberty Bay, outer	Starry Flounder	2.6	0.3	
WA00-0021	39-3736	Elliott Bay, northeast	English Sole	<LOQ		
WA00-0022	39-3735	Duamish River - East Wa	English Sole	2.5	0.2	
WA00-0023	39-3740	Port Ludlow	English Sole	<LOQ		
WA00-0024	39-9727	Hood Canal (north)	English Sole	<LOQ		
WA00-0025	39-9728	Port Gamble Bay	English Sole	0.6	0.1	Mean of 5
WA00-0030	39-3738	Hood Canal (south)	English Sole	<LOQ		
WA00-0031	40-3768	Port of Shelton	Shiner Perch	8.2	0.6	
WA00-0032	40-3742-1	Budd Inlet	Speckled Sanddab	<LOD		
WA00-0032	40-3742-2	Budd Inlet	Speckled Sanddab	<LOQ		
WA00-0033	39-9724-1	Port of Olympia	Pacific Staghorn Sculpin	3.8	0.3	
WA00-0033	40-3745-1	Port of Olympia	Shiner Perch	17.8	1.1	
WA00-0033	40-3745-2	Port of Olympia	Shiner Perch	7.2	0.5	
WA00-0033	39-3724-2	Port of Olympia	Pacific Staghorn Sculpin	2.2	0.2	Mean of 3
WA00-0034	40-3741	Case Inlet	Starry Flounder	1.6	0.1	
WA00-0035	37-9706	East Anderson Island/	Dover Sole	1.8	0.2	
WA00-0036	40-3742	Hale Passage	English Sole	1.2	0.1	
WA00-0037	40-3759	Gig Harbor	English Sole	1.7	0.1	
WA00-0038	37-9707	Colvos Passage	Rock Sole	0.3	0.1	

Table 4. TCDD equivalents (pg/g) of EMAP samples taken in Puget Sound and the Straight of Georgia

Station	Composite ID	Sampling Site	Species	TCDD-EQ (pg/g)	SD	
WA00-0038	37-9703	Colvos Passage	Dover Sole	0.4	0.1	Mean of 5
WA00-0040	37-9714	S.E. Commencement Bay	Dover Sole	37.3	8.0	
WA00-0041	37-3748	Hylebos Waterway	English Sole	2.2	0.2	
WA00-0042	40-3762-1	Puget Sound	Rock Sole	<LOQ		
WA00-0042	40-3762-2	Puget Sound	Rock Sole	<LOQ		
WA00-0045	37-9719	Puget Sound	Dover Sole	2.0	0.4	
WA00-0046	39-9730	Puget Sound	Rock Sole	<LOQ		Mean of 3
WA00-0047	40-3757	Puget Sound	English Sole	<LOQ		
WA00-0048	39-9732	Puget Sound	English Sole	<LOD		
WA00-0049	39-9723	Puget Sound	English Sole	<LOD		
WA00-0050	39-9733	Puget Sound	English Sole	<LOQ		
WA00-0054	39-9722	Puget Sound	Starry Flounder	<LOD		
WA00-0055	40-3752	West Sound	English Sole	10.8	2.3	
WA00-0056	40-3765	San Juan Channel	Rock Sole	<LOQ		
WA00-0058	39-3736	East Sound	English Sole	3.6	0.4	Mean of 5
WA00-0059	40-3761	San Juan Channel	Pacific Tomcod	<LOQ		
WA00-0066	39-9731	Puget Sound	English Sole	4.5	0.7	
WA00-0068	39-3734	Puget Sound	English Sole	<LOQ		
WA00-0069	37-9708	Puget Sound	Rock Sole	<LOD		
WA00-0069	39-9725	Puget Sound	English Sole	1.8	0.3	
WA00-0070	37-9718	Puget Sound	Rock Sole	4.0	0.5	
WA00-0071	37-9709	Puget Sound	Dover Sole	1.0	0.2	Mean of 3

Table 5. TCDD equivalents (pg/g) of EMAP samples taken in the lower Columbia River

Station	Composite ID	Sampling Site	Species	TCDD-EQ (pg/g)	SD	
OR2000-0002	ODEQ002A	Columbia River, Upstream of Wallace Island	Northern Pikeminnow	1.8	0.2	
OR2000-0003	ODEQ003A	Columbia River, North of Crims Island	Northern Pikeminnow	3.4	0.2	
OR2000-0003	ODEQ003B	Columbia River, North of Crims Island	Northern Pikeminnow	0.7	0.1	
OR2000-0004	ODEQ004A	Columbia River, North of Wallace Island	Starry Flounder	2.3	0.2	mean of 3
OR2000-0005	ODEQ005A	Columbia River, Barlow Pt.	Northern Pikeminnow	1.7	0.2	
OR2000-0005	ODEQ005B	Columbia River, Barlow Pt.	Peamouth	7.1	0.5	
OR2000-0007	ODEQ007A	Columbia River, NE of Slaughter's Dike	White Sturgeon	4.2	0.6	
OR2000-0007	ODEQ007B	Columbia River, NE of Slaughter's Dike	Peamouth	1.1	0.6	
OR2000-0009	ODEQ009B	Columbia River, East Ranier	Peamouth	6.2	0.8	
OR2000-0010	ODEQ010A	Columbia River, Prescott Beach	White Sturgeon	6.7	0.8	
OR2000-0011	ODEQ011A	Columbia River, North Kalama	White Sturgeon	3.0	0.4	mean of 5
OR2000-0012	ODEQ012A	Columbia River, Deer Island Slough	White Sturgeon	<LOQ		
OR2000-0013	ODEQ013A	Columbia River, North St. Helens	White Sturgeon	<LOQ		
OR2000-0014	ODEQ014A	Columbia River, South St. Helens (North of Sand Island)	Peamouth	2.2	0.5	
OR2000-0015	ODEQ015A	Columbia River, Warrior Rock (North Sauvie Island)	Northern Pikeminnow	2.2	0.3	
OR2000-0016	ODEQ016D	Columbia River, Kelley Pt.	White Sturgeon	2.8	0.3	
OR2000-0017	ODEQ017A	Columbia River, Downstream End of Hayden Island	Peamouth	<LOQ		
OR2000-0019	ODEQ019A	Columbia River, North of West Lemon Island	Peamouth	<LOQ		
OR2000-0020	ODEQ020A	Columbia River, SW of Lemon Island (PDX Tower to South)	White Sturgeon	1.9	0.2	
OR2000-0020	ODEQ020B	Columbia River, SW of Lemon Island (PDX Tower to South)	White Sturgeon	4.7	1.0	
OR2000-0021	ODEQ021A	Columbia River, N. Channel-Mid Government Island	Peamouth	1.9	0.1	
OR2000-0021	ODEQ021B	Columbia River, N. Channel-Mid Government Island	White Sturgeon	1.2	0.1	

Table 5. TCDD equivalents (pg/g) of EMAP samples taken in the lower Columbia River

Station	Composite ID	Sampling Site	Species	TCDD-EQ (pg/g)	SD	
OR2000-0021	ODEQ021C	Columbia River, N. Channel-Mid Government Island	Northern Pikeminnow	0.9	0.1	mean of 3
OR2000-0022	ODEQ022A	Columbia River, East of Sandy River Mouth (1)	Peamouth	5.8	0.6	
OR2000-0023	ODEQ023A	Columbia River, East of Sandy River Mouth (2)	White Sturgeon	0.9	0.1	
OR2000-0023	ODEQ023B	Columbia River, East of Sandy River Mouth (2)	Northern Pikeminnow	2.7	0.2	
OR2000-0024	ODEQ024A	Columbia River, Beacon Rock	White Sturgeon	1.2	0.1	
OR2000-0025	ODEQ025A	Columbia River, East of Franz Lake	White Sturgeon	4.3	0.8	
OR2000-0026	ODEQ026A	Columbia River, Norht of Reed Island	White Sturgeon	<LOQ		
OR2000-0027	ODEQ027A (composite of 0027 & 0030)	Columbia River, East of Phoca Rock (near boat ramp)	White Sturgeon	4.7	0.4	
OR2000-0027	ODEQ027B	Columbia River, East of Phoca Rock (near boat ramp)	Peamouth	15.7	1.1	
OR2000-0028	ODEQ028A	Columbia River, South of Reed Island	Peamouth	6.5	0.5	
OR2000-0028	ODEQ028B	Columbia River, South of Reed Island	White Sturgeon	6.3	0.5	mean of 5
OR2000-0028	ODEQ028C	Columbia River, South of Reed Island	Northern Pikeminnow	30.0	6.6	
OR2000-0029	ODEQ029A	Columbia River, East of Reed Island	Starry Flounder	7.3	0.5	
OR2000-0029	ODEQ029B	Columbia River, East of Reed Island	Peamouth	4.4	0.3	
OR2000-0030	ODEQ030A	Columbia River, SW of Skamania I	Peamouth	8.4	1.2	mean of 3
OR2000-0030	ODEQ030B	Columbia River, SW of Skamania I	White Sturgeon	<LOQ		
OR2000-0031	ODEQ031A	Columbia River Mouth: west of Jetty A	Pacific Staghorn Sculpin	<LOD		
OR2000-0032	ODEQ032A	Columbia River, Sw of Sand Island Tower	Pacific Staghorn Sculpin	1.1	0.5	
OR2000-0033	ODEQ033A	Columbia River, East of Clatsop Spit	Pacific Staghorn Sculpin	<LOQ		
OR2000-0033	ODEQ033B	Columbia River, East of Clatsop Spit	English Sole	249.5	46.7	
OR2000-0034	ODEQ034A	Columbia River, "North Shore" (E. of N. Astoria Bridge)	Pacific Staghorn Sculpin	<LOQ		

Table 5. TCDD equivalents (pg/g) of EMAP samples taken in the lower Columbia River

Station	Composite ID	Sampling Site	Species	TCDD-EQ (pg/g)	SD	
OR2000-0035	ODEQ035A	Columbia River, West of Grays Pt.	Pacific Staghorn Sculpin	4.1	1.1	
OR2000-0036	ODEQ036A	Columbia River, Mid-river between Hammond, OR and Chinook Pt., WA	Pacific Staghorn Sculpin	3.8	0.8	mean of 5
OR2000-0037	ODEQ037A	Columbia River, East of N. Astoria Bridge	Pacific Staghorn Sculpin	<LOD		
OR2000-0038	ODEQ038A	Columbia River, Pt. Ellice (SW of North Astoria Gridge)	Pacific Staghorn Sculpin	<LOQ		
OR2000-0039	ODEQ039A	Columbia River, North of Taylor Sands	Pacific Staghorn Sculpin	<LOD		
OR2000-0040	ODEQ040A	Columbia River, North of Rice Island	White Sturgeon	8.8	0.9	
OR2000-0041	ODEQ041A	Columbia River, Desdemona Sands (Downstream of Astoria Bridge)	Pacific Staghorn Sculpin	2.8	0.5	
OR2000-0042	ODEQ042A	Columbia River, West of Taylor Sands	English Sole	<LOQ		
OR2000-0043	ODEQ043A	Columbia River, SW of Rice Island	Pacific Staghorn Sculpin	2.8	0.4	Mean of 5
OR2000-0044	ODEQ044A	Columbia River, Altoona	Pacific Staghorn Sculpin	<LOQ		
OR2000-0045	ODEQ045B	Columbia River, East of Jim Crow Sands; West of Jim Crow Pt.	Pacific Staghorn Sculpin	4.3	0.7	
OR2000-0046	ODEQ046B	Columbia River, NW of Tongue Pt. Coast Guard Station	Pacific Staghorn Sculpin	4.0	0.7	
OR2000-0046	ODEQ046C	Columbia River, NW of Tongue Pt. Coast Guard Station	Pacific Staghorn Sculpin	4.3	0.6	
OR2000-0047	ODEQ047A	Columbia River, North of Green Marsh	Pacific Staghorn Sculpin	<LOD		
OR2000-0048	ODEQ048A	Columbia River, North of Fitzpatrick	Pacific Staghorn Sculpin	4.7	0.7	Mean of 3

Table 6. TCDD equivalents (pg/g) of EMAP samples taken in San Francisco Bay, CA

Station	Composite ID	Sampling Site	Species	TCDD-EQ (pg/g)	SD	
CA00-0001	01A	San Francisco Bay, CA	Pacific Staghorn Sculpin	<LOQ		
CA00-0002	02A	San Francisco Bay, CA	English Sole	<LOQ		
CA00-0002	02B	San Francisco Bay, CA	Pacific Staghorn Sculpin	4.5	0.5	
CA00-0003	03A	San Francisco Bay, CA	English Sole	<LOQ		
CA00-0003	03B	San Francisco Bay, CA	Pacific Staghorn Sculpin	<LOQ		
CA00-0004	04A	San Francisco Bay, CA	Starry Flounder	8.2	0.9	
CA00-0006	06A	San Francisco Bay, CA	CA Halibut	3.0	0.3	
CA00-0012	12A	San Francisco Bay, CA	CA Halibut	2.9	0.2	
CA00-0014	14A	San Francisco Bay, CA	Pacific Staghorn Sculpin	1.9	0.2	
CA00-0015	15A	San Francisco Bay, CA	Pacific Staghorn Sculpin	4.4	0.6	
CA00-0016	16A	San Francisco Bay, CA	English Sole	9.5	1.3	Mean of 5
CA00-0016	16B	San Francisco Bay, CA	Pacific Staghorn Sculpin	<LOD		
CA00-0017	17A	San Francisco Bay, CA	CA Halibut	1.7	0.2	
CA00-0017	17B	San Francisco Bay, CA	Pacific Staghorn Sculpin	19.7	0.3	
CA00-0019	19A	San Francisco Bay, CA	Pacific Staghorn Sculpin	3.2	0.3	
CA00-0024	24A	San Francisco Bay, CA	Starry Flounder	3.1	0.4	
CA00-0025	25A	San Francisco Bay, CA	Starry Flounder	68.5	11.2	
CA00-0027	27A	San Francisco Bay, CA	Pacific Staghorn Sculpin	1.6	0.2	
CA00-0029	29A	San Francisco Bay, CA	CA Halibut	3.0	0.3	Mean of 2
CA00-0030	30A	San Francisco Bay, CA	Pacific Staghorn Sculpin	1.6	0.2	
CA00-0030	30B	San Francisco Bay, CA	Starry Flounder	3.1	0.3	
CA00-0031	31A	San Francisco Bay, CA	CA Halibut	6.0	0.5	
CA00-0031	31B	San Francisco Bay, CA	Starry Flounder	5.4	0.5	
CA00-0032	32A	San Francisco Bay, CA	CA Halibut	10.5	1.1	
CA00-0032	32B	San Francisco Bay, CA	Pacific Staghorn Sculpin	3.9	0.6	Mean of 5
CA00-0033	33A	San Francisco Bay, CA	CA Halibut	4.3	0.5	
CA00-0034	34A	San Francisco Bay, CA	CA Halibut	12.1	0.9	
CA00-0034	34B	San Francisco Bay, CA	Pacific Staghorn Sculpin	7.1	0.6	
CA00-0035	35A	San Francisco Bay, CA	CA Halibut	<LOQ		
CA00-0035	35B	San Francisco Bay, CA	Pacific Staghorn Sculpin	4.7	0.4	
CA00-0036	36A	San Francisco Bay, CA	English Sole	4.3	0.5	Mean of 3
CA00-0036	36B	San Francisco Bay, CA	Pacific Staghorn Sculpin	3.9	0.4	
CA00-0037	37A	San Francisco Bay, CA	CA Halibut	6.7	0.5	
CA00-0037	37B	San Francisco Bay, CA	Pacific Staghorn Sculpin	<LOD		
CA00-0038	38A	San Francisco Bay, CA	Pacific Staghorn Sculpin	1.8	0.4	
CA00-0042	42A	San Francisco Bay, CA	CA Halibut	3.1	0.4	
CA00-0044	44A	San Francisco Bay, CA	CA Halibut	<LOQ		
CA00-0044	44B	San Francisco Bay, CA	Pacific Staghorn Sculpin	<LOD		
CA00-0044	44C	San Francisco Bay, CA	Starry Flounder	1.4	0.3	Mean of 5

Table 6. TCDD equivalents (pg/g) of EMAP samples taken in San Francisco Bay, CA

Station	Composite ID	Sampling Site	Species	TCDD-EQ (pg/g)	SD
CA00-0045	45A	San Francisco Bay, CA	CA Halibut	5.3	0.5
CA00-0046	46A	San Francisco Bay, CA	Starry Flounder	17.3	4.8
CA00-0048	48A	San Francisco Bay, CA	CA Halibut	5.1	1.2
CA00-0049	49A	San Francisco Bay, CA	CA Halibut	7.0	0.7
CA00-0050	50A	San Francisco Bay, CA	CA Halibut	2.6	0.3

Table 7. Station mean TCDD equivalents (pg/g) of EMAP samples taken in Puget Sound and the Straight of Georgia

Station	Species	Mean TCDD-EQ (pg/g)	SD	*n*	range
WA00-0001	Rock Sole/Starry Flounder	1.89	0.65	3	1.5 - 2.7
WA00-0002	English Sole	0.55*	0.0	1	
WA00-0003	English Sole	0.3*	0.0	1	
WA00-0004	English Sole/Starry Flounder	2.35	1.69	3	<LOQ - 4.1
WA00-0005	English Sole	1.5	0.2	1	
WA00-0007	Starry Flounder	0.3*	0.0	1	
WA00-0008	English Sole	0.55*	0.0	1	
WA00-0009	English Sole	0.25**	0.0	1	
WA00-0010	English Sole	0.15**	0.0	1	
WA00-0011	English Sole	0.3*	0.0	1	
WA00-0013	English Sole	1.2	0.3	1	
WA00-0014	English Sole	2.1	0.3	1	
WA00-0017	English Sole	2.7	0.5	1	
WA00-0018	English Sole	3.1	0.4	1	
WA00-0019	English Sole	3.5	0.4	1	
WA00-0020	Starry Flounder	2.6	0.3	1	
WA00-0021	English Sole	0.3*	0.0	1	
WA00-0022	English Sole	2.5	0.2	1	
WA00-0023	English Sole	0.3*	0.0	1	
WA00-0024	English Sole	0.3*	0.0	1	
WA00-0025	English Sole	0.6	0.1	1	
WA00-0030	English Sole	0.3*	0.0	1	
WA00-0031	Shiner Perch	8.2	0.6	1	
WA00-0032	Speckled Sanddab	0.3*	0.0	2	<LOD - <LOQ
WA00-0033	Pacific Staghorn Sculpin/Shiner Perch	5.66	7.01	4	2.2 - 17.8
WA00-0034	Starry Flounder	1.6	0.1	1	
WA00-0035	Dover Sole	1.8	0.2	1	
WA00-0036	English Sole	1.2	0.1	1	
WA00-0037	English Sole	1.7	0.1	1	
WA00-0038	Rock Sole/Dover Sole	0.35	0.10	2	0.3 - 0.4
WA00-0040	Dover Sole	37.3	8.0	1	
WA00-0041	English Sole	2.2	0.2	1	
WA00-0042	Rock Sole	0.3*	0.00	2	<LOQ - <LOQ
WA00-0045	Dover Sole	1.99	0.38	1	
WA00-0046	Rock Sole	0.97*	0.00	1	
WA00-0047	English Sole	0.97*	0.00	1	
WA00-0048	English Sole	0.5**	0.00	1	
WA00-0049	English Sole	0.5**	0.00	1	

Table 7. Station mean TCDD equivalents (pg/g) of EMAP samples taken in Puget Sound and the Straight of Georgia

Station	Species	Mean TCDD-EQ (pg/g)	SD	*n*	range
WA00-0050	English Sole	0.97*	0.00	1	
WA00-0054	Starry Flounder	0.5**	0.00	1	
WA00-0055	English Sole	10.76	2.31	1	
WA00-0056	Rock Sole	0.97*	0.00	1	
WA00-0058	English Sole	3.61	0.45	1	
WA00-0059	Pacific Tomcod	0.97*	0.00	1	
WA00-0066	English Sole	4.51	0.68	1	
WA00-0068	English Sole	0.97*	0.00	1	
WA00-0069	Rock Sole/English Sole	0.97*	0.00	2	<LOD - 1.8
WA00-0070	Rock Sole	4.00	0.55	1	
WA00-0071	Dover Sole	0.96	0.19	1	

* 1/2 LOQ

** 1/2 LOD

Table 8. Station mean TCDD Equivalents (pg/g) of EMAP samples taken in the lower Columbia River

Station	Species	Mean TCDD-EQ (pg/g)	SD	*n*	range
OR2000-0002	Northern Pikeminnow	1.8	0.2	1	
OR2000-0003	Northern Pikeminnow	1.6	1.9	2	0.7 - 3.4
OR2000-0004	Starry Flounder	2.3	0.2	1	
OR2000-0005	Northern Pikeminnow/Peamouth	3.5	3.8	2	1.7 - 7.1
OR2000-0007	White Sturgeon/Peamouth	2.1	2.2	2	1.1 - 4.2
OR2000-0009	Peamouth	6.2	0.8	1	
OR2000-0010	White Sturgeon	6.7	0.8	1	
OR2000-0011	White Sturgeon	3.0	0.4	1	
OR2000-0012	White Sturgeon	0.6*	0.0	1	
OR2000-0013	White Sturgeon	0.6*	0.0	1	
OR2000-0014	Peamouth	2.2	0.5	1	
OR2000-0015	Northern Pikeminnow	2.2	0.3	1	
OR2000-0016	White Sturgeon	2.8	0.3	1	
OR2000-0017	Peamouth	0.6*	0.0	1	
OR2000-0019	Peamouth	0.6*	0.0	1	
OR2000-0020	White Sturgeon	3.0	1.9	2	1.9 - 4.7
OR2000-0021	Peamouth/White Sturgeon/Northern Pikeminnow	1.2	0.5	3	0.9 - 1.9
OR2000-0022	Peamouth	5.8	0.6	1	
OR2000-0023	White Sturgeon/Northern Pikeminnow	1.5	1.3	2	0.9 - 2.7
OR2000-0024	White Sturgeon	1.2	0.1	1	
OR2000-0025	White Sturgeon	4.3	0.8	1	
OR2000-0026	White Sturgeon	0.3*	0.0	1	
OR2000-0027	White Sturgeon/Peamouth	8.6	7.8	2	4.7 - 15.7
OR2000-0028	Peamouth/White Sturgeon/Northern Pikeminnow	10.7	13.6	3	6.3 - 30.0
OR2000-0029	Starry Flounder/Peamouth	5.7	2.1	2	4.4 - 7.3

Table 8. Station mean TCDD Equivalents (pg/g) of EMAP samples taken in the lower Columbia River

Station	Species	Mean TCDD-EQ (pg/g)	SD	*n*	range
R2000-0030	Peamouth/White Sturgeon	3.2	1.3	2	<LOQ - 8.4
OR2000-0031	Pacific Staghorn Sculpin	1.05**	0.0	1	
OR2000-0032	Pacific Staghorn Sculpin	1.1	0.5	1	
OR2000-0033	Pacific Staghorn Sculpin/English Sole	23.9	174.8	2	<LOQ - 249.5
OR2000-0034	Pacific Staghorn Sculpin	0.5*	0.0	1	
OR2000-0035	Pacific Staghorn Sculpin	4.1	1.1	1	
OR2000-0036	Pacific Staghorn Sculpin	3.9	0.8	1	
OR2000-0037	Pacific Staghorn Sculpin	1.05**	0.0	1	
OR2000-0038	Pacific Staghorn Sculpin	1.85*	0.0	1	
OR2000-0039	Pacific Staghorn Sculpin	1.05**	0.0	1	
OR2000-0040	White Sturgeon	8.8	0.9	1	
OR2000-0041	Pacific Staghorn Sculpin	2.8	0.5	1	
OR2000-0042	English Sole	0.3*	0.0	1	
OR2000-0043	Pacific Staghorn Sculpin	2.8	0.4	1	
OR2000-0044	Pacific Staghorn Sculpin	1.85*	0.0	1	
OR2000-0045	Pacific Staghorn Sculpin	4.3	0.7	1	
OR2000-0046	Pacific Staghorn Sculpin	4.2	0.2	2	4.0 - 4.3
OR2000-0047	Pacific Staghorn Sculpin	1.05**	0.0	1	
OR2000-0048	Pacific Staghorn Sculpin	4.7	0.7	1	

* 1/2 LOQ

** 1/2 LOD

Table 9. Station mean TCDD equivalents (pg/g) of EMAP samples taken in San Francisco Bay, CA

Station	Species	Mean TCDD-EQ (pg/g)	SD	*n*	Range
CA00-0001	Pacific Staghorn Sculpin	1.2*	0.0	1	
CA00-0002	English Sole/Pacific Staghorn Sculpin	2.5	2.2	2	<LOQ - 4.5
CA00-0003	English Sole/Pacific Staghorn Sculpin	1.2*	0.0	2	<LOQ - <LOQ
CA00-0004	Starry Flounder	8.2	0.9	1	
CA00-0006	CA Halibut	3.0	0.3	1	
CA00-0012	CA Halibut	2.9	0.2	1	
CA00-0014	Pacific Staghorn Sculpin	1.9	0.2	1	
CA00-0015	Pacific Staghorn Sculpin	4.4	0.6	1	
CA00-0016	English Sole/Pacific Staghorn Sculpin	1.9	6.5	2	<LOD - 9.5
CA00-0017	CA Halibut/Pacific Staghorn Sculpin	5.7	12.7	2	1.7 - 19.7
CA00-0019	Pacific Staghorn Sculpin	3.2	0.3	1	
CA00-0024	Starry Flounder	3.1	0.4	1	
CA00-0025	Starry Flounder	68.5	11.2	1	
CA00-0027	Pacific Staghorn Sculpin	1.6	0.2	1	
CA00-0029	CA Halibut	3.0	0.3	1	
CA00-0030	Pacific Staghorn Sculpin/Starry Flounder	2.2	1.1	2	1.6 - 3.1
CA00-0031	CA Halibut/Starry Flounder	5.7	0.4	2	5.4 - 6.0
CA00-0032	CA Halibut/Pacific Staghorn Sculpin	6.4	4.7	2	3.9 - 10.5
CA00-0033	CA Halibut	4.3	0.5	1	
CA00-0034	CA Halibut/Pacific Staghorn Sculpin	9.2	3.6	2	7.1 - 12.1
CA00-0035	CA Halibut/Pacific Staghorn Sculpin	2.6	2.3	2	<LOQ - 4.7
CA00-0036	English Sole/Pacific Staghorn Sculpin	4.1	0.3	2	3.9 - 4.3
CA00-0037	CA Halibut/Pacific Staghorn Sculpin	2.1	4.3	2	<LOD - 6.7
CA00-0038	Pacific Staghorn Sculpin	1.8	0.4	1	
CA00-0042	CA Halibut	3.1	0.4	1	
CA00-0044	CA Halibut/Pacific Staghorn Sculpin/Starry Flounder	0.2**	0.0	3	<LOD - 1.4
CA00-0045	CA Halibut	5.3	0.5	1	
CA00-0046	Starry Flounder	17.3	4.8	1	
CA00-0048	CA Halibut	5.1	1.2	1	
CA00-0049	CA Halibut	6.9	0.7	1	
CA00-0050	CA Halibut	2.5	0.2	1	

* 1/2 LOQ

** 1/2 LOD

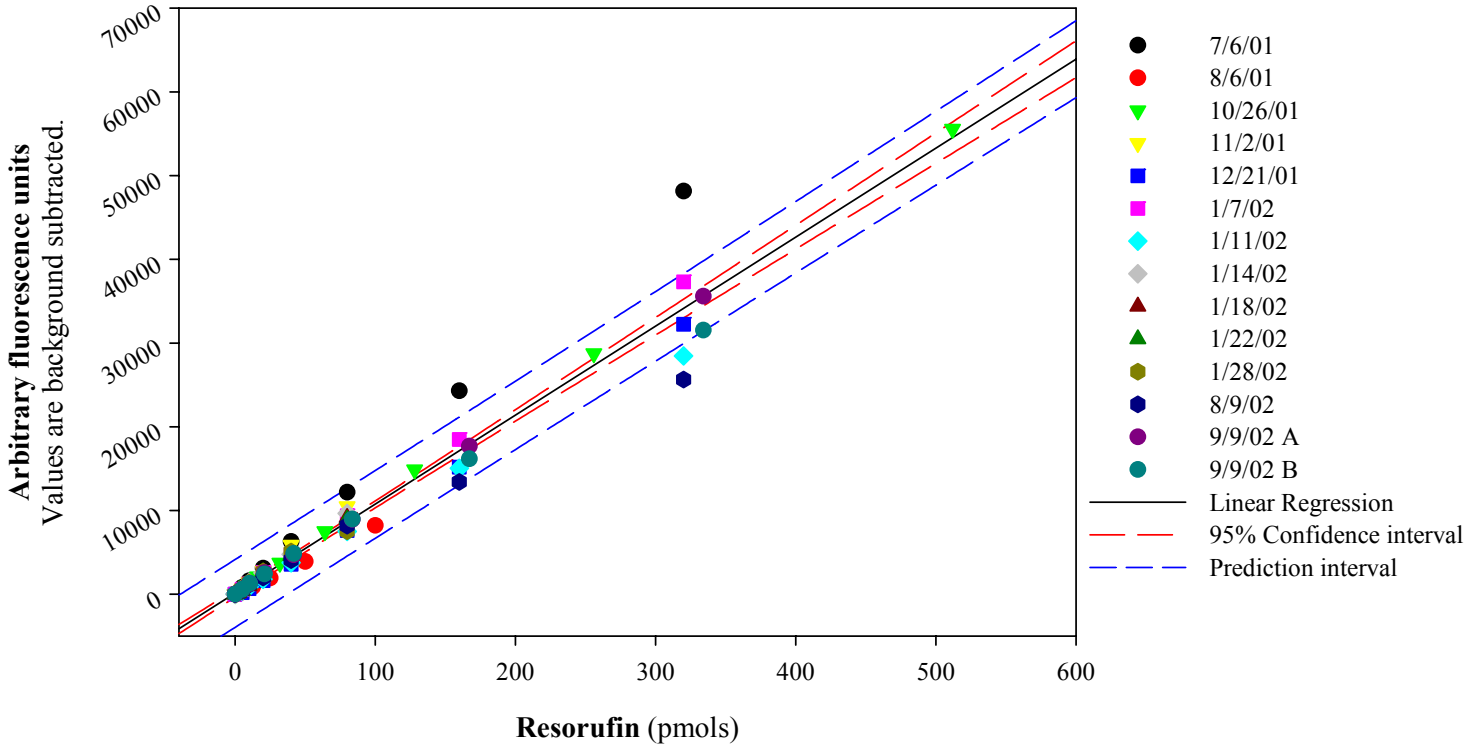

Figure 1. Resorufin standard graph. All resorufin standards that were used for EMAP sample analysis.

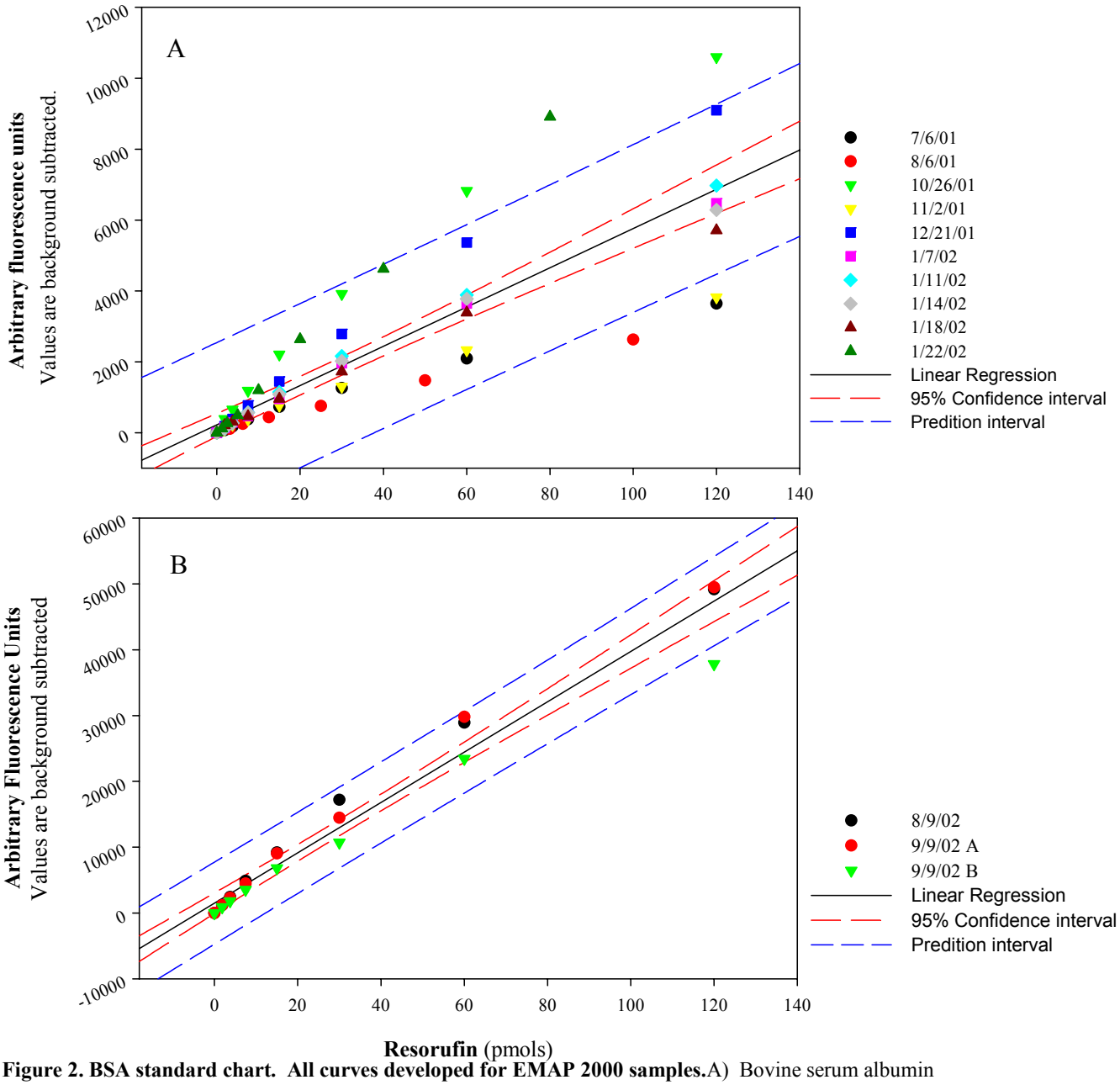

Figure 2. BSA standard chart. All curves developed for EMAP 2000 samples.A) Bovine serum albumin (BSA) standard curves determined using 360 nm excitation and 460 emission filters. B) BSA standard curves determined using 400 nm excitation and 460 emission filters.

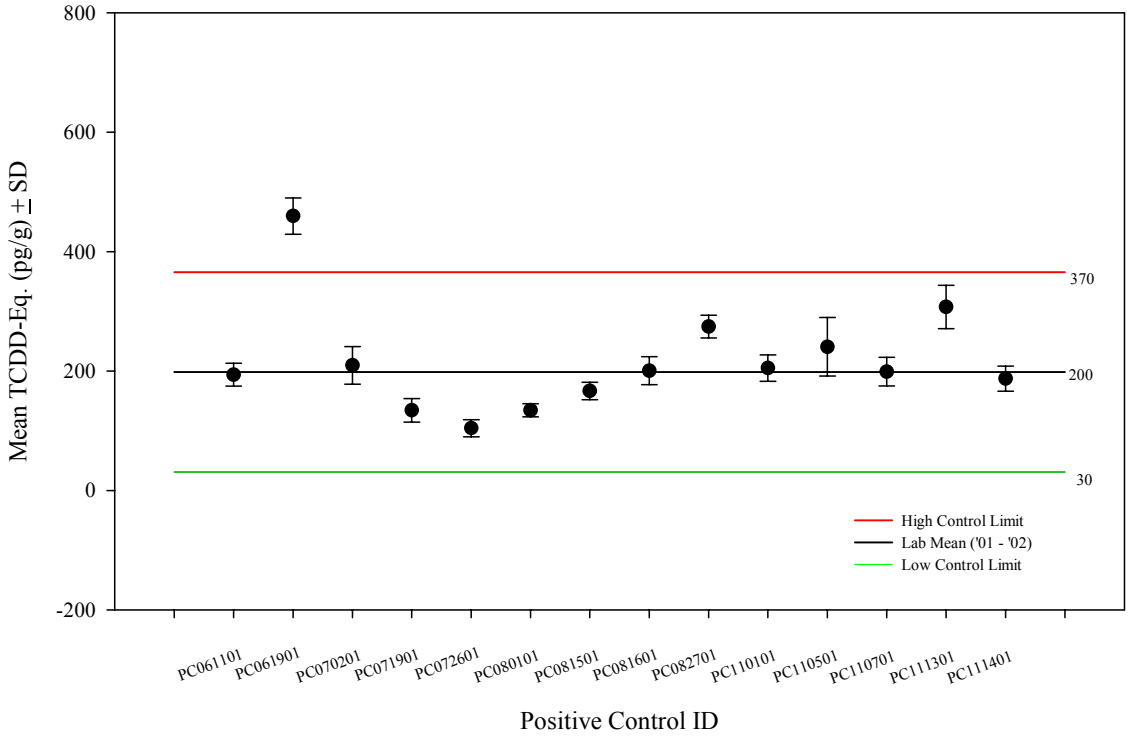

Figure 3. Positive Control, Control Chart All Saginaw Bay carp positive control samples prepared with 2000 EMAP samples.

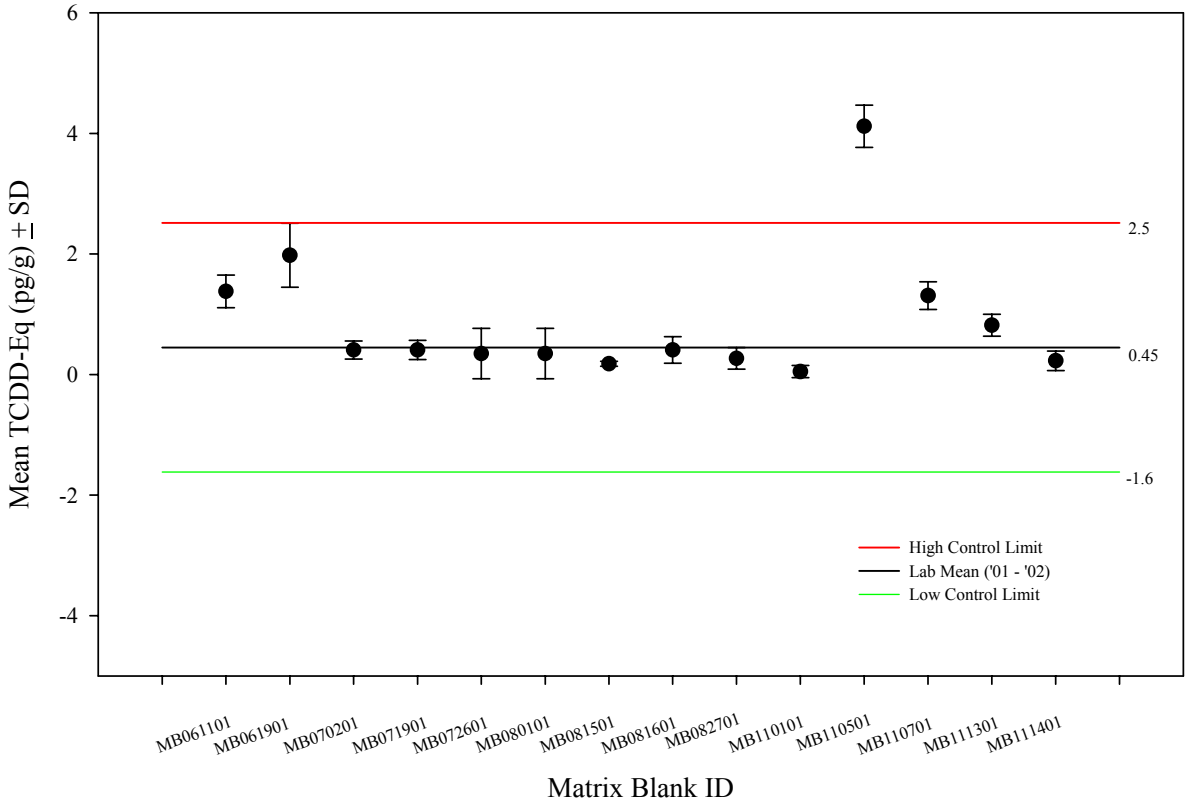

Figure 4. Matrix Blank Control Chart. All bluegill matrix blank samples prepared with 2000 EMAP samples are shown.

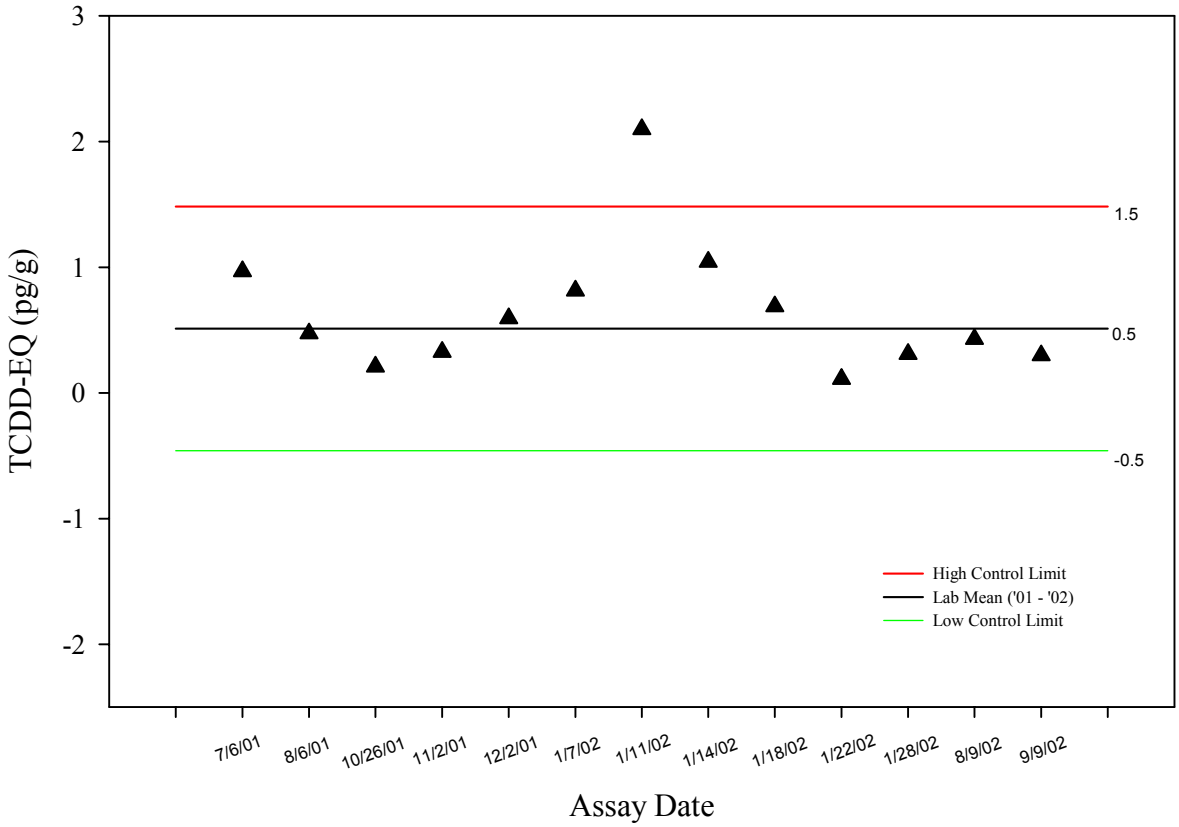

Figure 5. Limit of Detection. All LOD's determined for EROD analyses of 2000 EMAP samples.

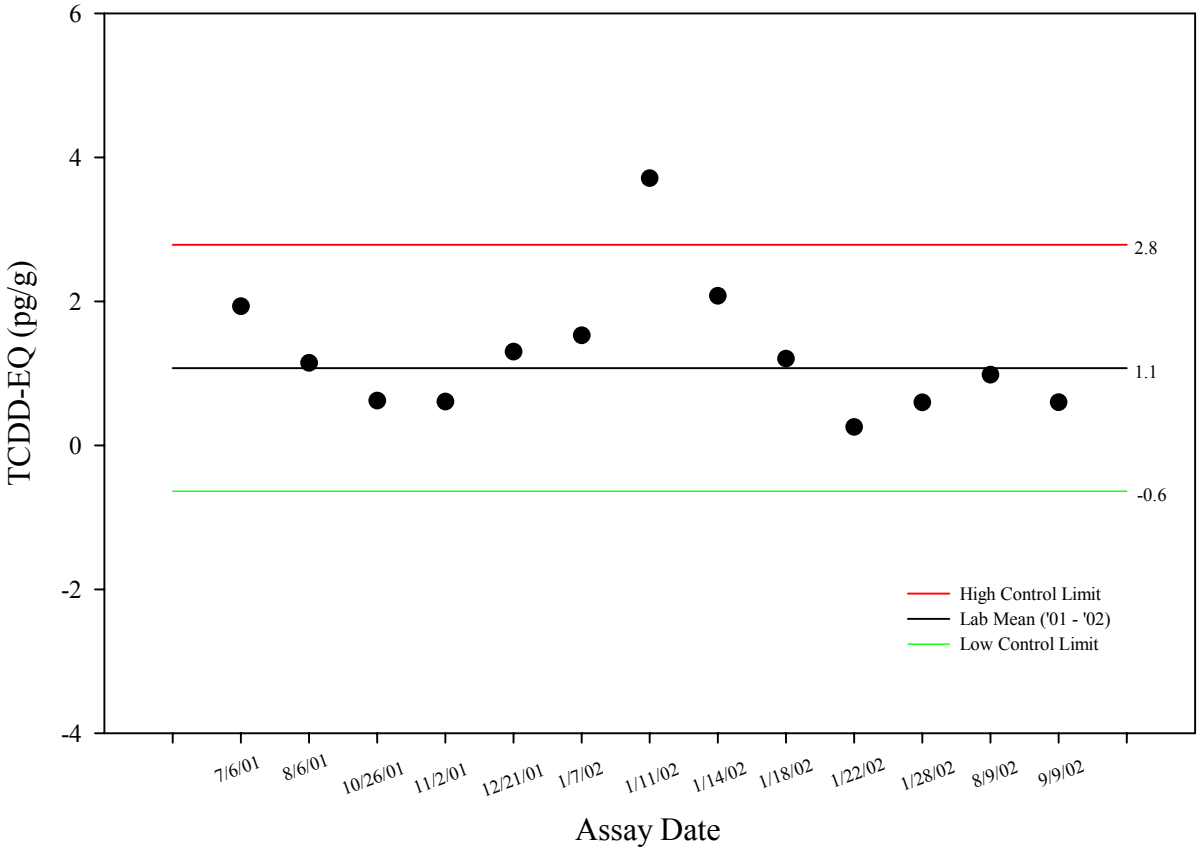

Figure 6. Limit of Quantitation. All LOQ's determined for EROD analyses of 2000 EMAP samples.

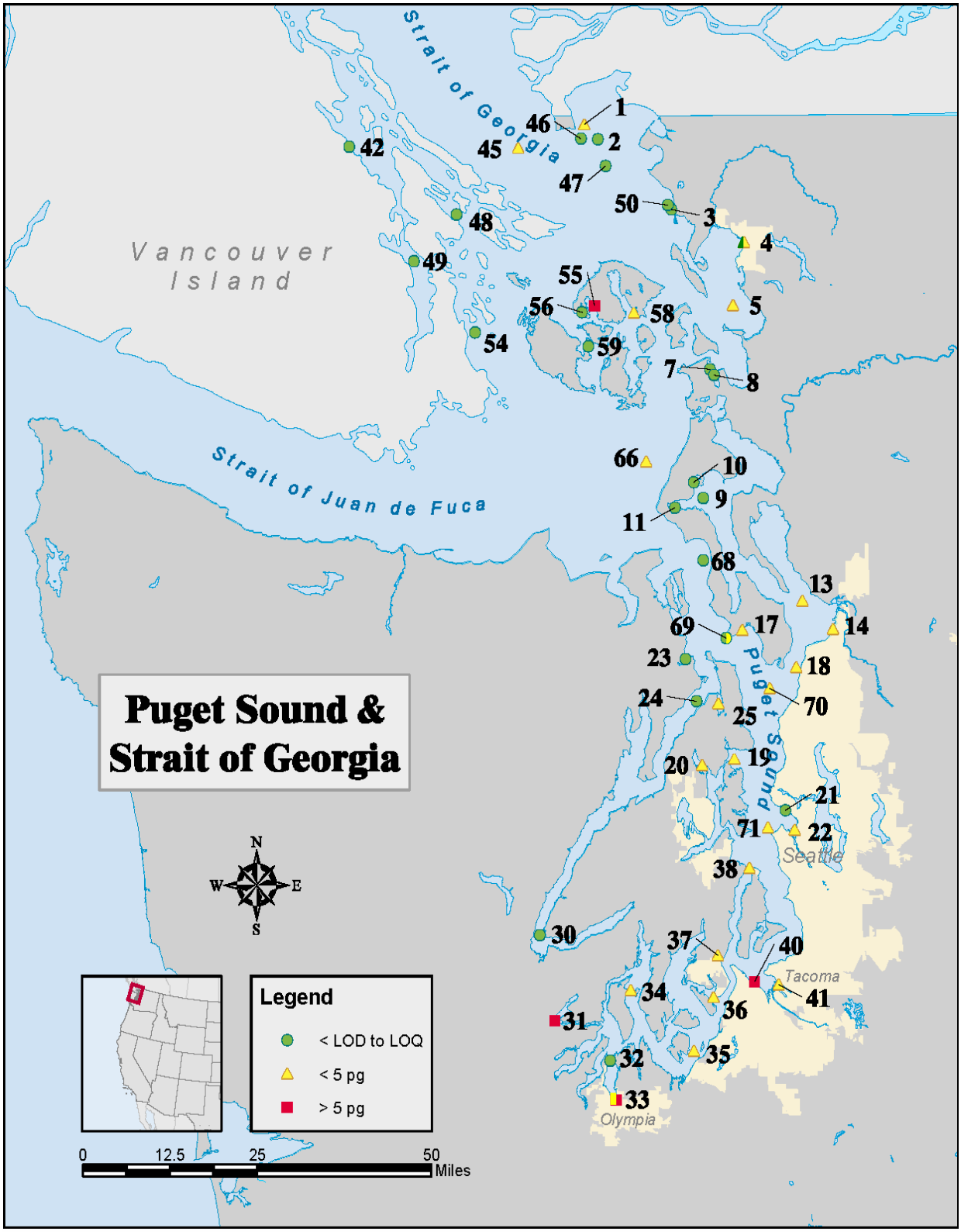

Figure 7. H4IIE bioassay-derived TCDD-EQ results for EMAP sites in Puget Sound and the Strait of Georgia.

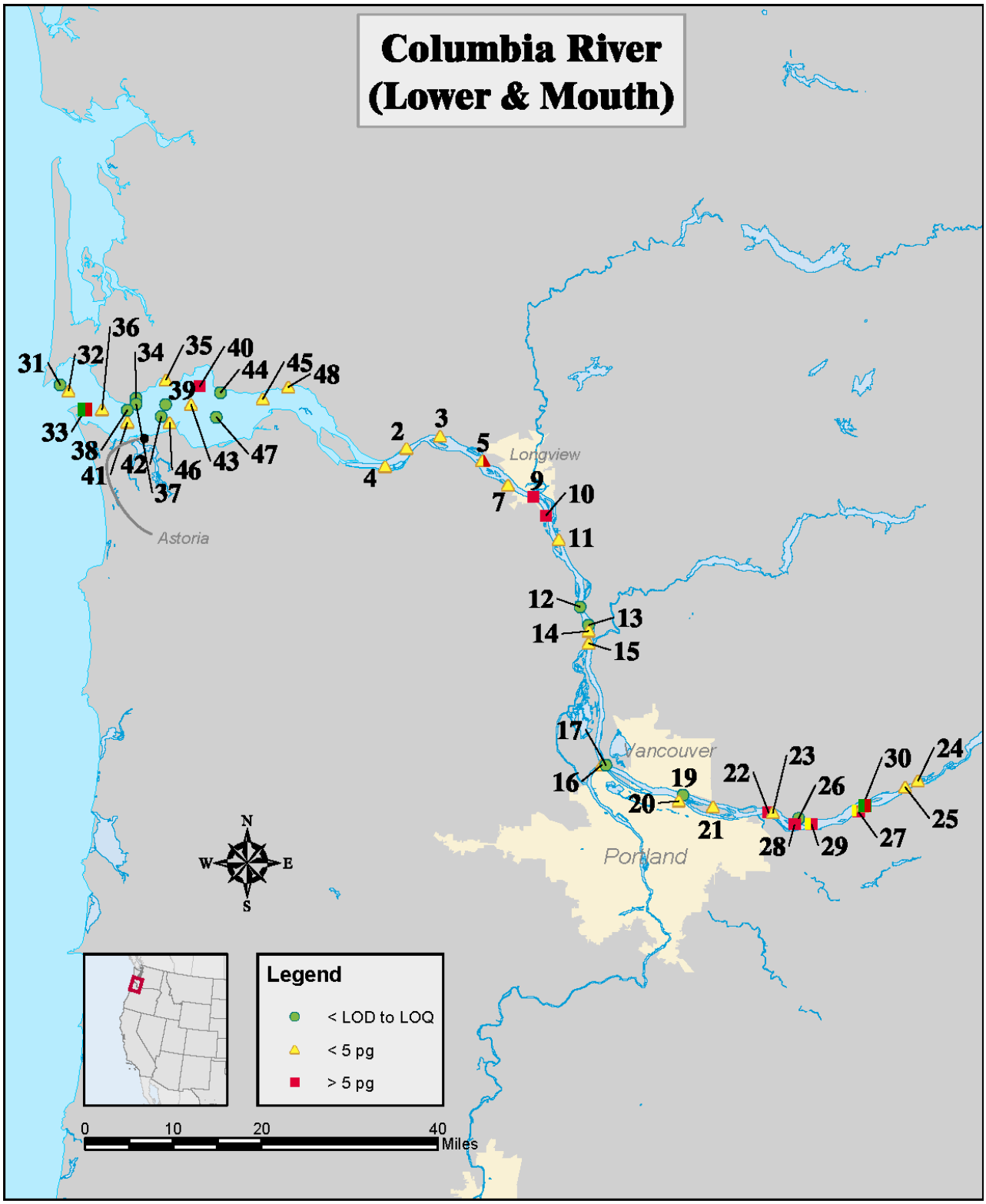

Figure 8. H4IIE bioassay-derived TCDD-EQ results for EMAP sites in the lower Columbia River.

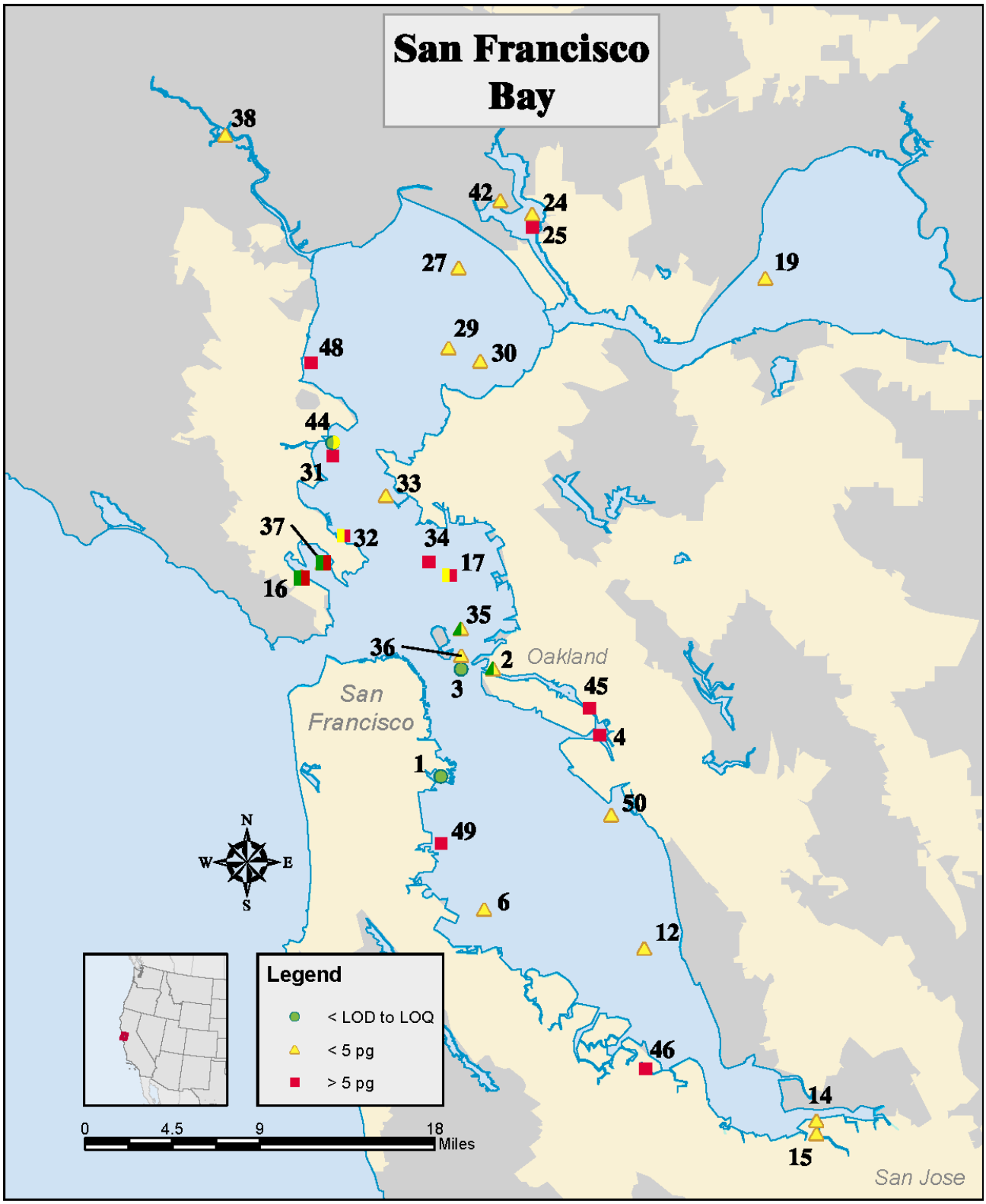

Figure 9. H4IIE bioassay-derived TCDD-EQ results for EMAP sites in San Francisco Bay.

Attachment 1

NBS-MSC SOPC5.194
Date Prepared: 2/7/94
Date Revised: 2/3/03

FOR USERS OTHER THAN ECRC STAFF, THIS IS FOR REFERENCE ONLY. THIS IS NOT A CITABLE DOCUMENT.

PROCEDURE FOR THE DETERMINATION OF 7-ETHOXYRESORUFIN-O-DEETHYLASE (EROD) ACTIVITY IN H4IIE AND PLHC-1 CELLS USING 96-WELL MICROTITER PLATES

I. General:

Planar halogenated hydrocarbons (PHHs) are a group of structurally similar chemicals that include polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs). PHHs are currently believed to cause toxicity in living cells by entering the cell, binding to a cytosolic protein, the aryl hydrocarbon receptor (AhR), which translocates into the nucleus and acts as a transcription factor for specific dioxin responsive elements (DREs). Genes associated with these DREs increase in expression resulting in increased production of a number of "drug-metabolizing" enzymes. One of these enzymes is Cytochrome P4501A1 (CYP1A1) and 7-ethoxyresorufin-O-deethylase (EROD) is one of a number of CYP1A1 accompanying enzymes. The H4IIE rat hepatoma cell line has excellent growth properties, low basal CYP1A1 activity and high degree of responsiveness towards PHHs. Therefore, the H4IIE cell line is well suited for use in the following bioassay procedure. By comparing H4IIE EROD production in cells exposed to TCDD to cells exposed to an extracted environmental sample the environmental samples can be screened for potential toxicity to a living system.

II. Equipment:

Incubator equipped with CO_2 regulator
Fluorometric multiple well plate reader (Cytofluor 4000, Millipore Corp.)
8-Channel repeating pipettors (20 µL, 200 µL, & 1250 µL)
Single channel pipettors (10 µL, 100 µL, & 200 µL)
Sterile pipet tips for above pipettors
96-well flat bottom microtiter plates (Nunc 96 ∆)
Temperature controlled water bath (calibrated to 25° C)
Timer
Sterile 50 ml screw-cap centrifuge tubes
Bench-top plate shaker

96 well plate washer (Bio-Rad model 1550) equipped with reservoir filled with fresh ultra pure H_2O
Tissue paper (i.e. Kim wipes)
Hemacytometer (cell counting chamber) w/ cover slip
Beaker, 500 mL
Sterile 100 mL beakers
Sterile 10 mL pipets
Automatic pipettor (should accommodate 1, 10, & 25 mL pipets)
Sterile reagent wells
300 mL round bottom, crimp cap vials (Sun-Sri), dosing vials
Rack for 300 µL round bottom vials set up for 8-channel pipettor

III. Chemicals/Reagents:

Phosphate Buffered Saline (PBS), pH 7.8 (for H4IIE cells)
Cortland's Balanced Salt Solution, pH 7.4 (for PLHC-1 cells)
0.01% Trypsin solution (for H4IIE cells)
0.005% Trypsin w/ 0.02% EDTA solution (for PLHC-1 cells)
Dulbelcos Modified Essential Medium (D-MEM) with 15% FBS for H4IIE cells and 10% FBS for PLHC-1 cells
~2 mL per plate of 5 µM Ethoxyresorufin (ER) at 25° C
~2 mL per plate of 5 mM NADPH at 25° C
Phosphate Buffered Saline (PBS), pH 7.8 with 80 µM Dicumarol
Resorufin standards 0, 5, 10, 20, 40, 80, 160, and 320 pmol per 20 µl
BSA Standards 0, 7.5, 15, 30, 45, 60, 90 and 120 µg per 20 µl
~2 mL per plate of 1.08 µM Fluorescamine/20 µl solution (in acetone)
~15 mL per plate of Sodium Phosphate buffer (pH 8).
Note: See SOP Attachment for reagent preparation and storage.

IV. Procedures:

A. Seeding of microtiter plates:

Note: This procedure is performed under a Biological, Class II, and Type B2 hood. Fire all openings & caps before replacing caps!

1. Turn on hood & allow to equilibrate for 10 min.
2. Put on latex gloves.
3. Prepare Laminar Flow Hood by wiping down working area w/ 70% EtOH.

4. Get all solutions needed out of refrigerator (PBS, 0.01 uM Trypsin & D-MEM 15% F.B.S. for H4IIE or Cortland's buffered salt, 0.005 uM Trypsin w/ EDTA & D-MEM 10% F.B.S. for PLHC).
5. Gather all supplies needed: beaker for liquid waste, sterile pipets, auto pipettor, sterile beaker(s), sterile 50 mL centrifuge tubes, hemacytometer & cover slip, 10 ul pipettor & sterile tips, 1250 µL 8-channel pipettor, sterile reagent well(s), sterile microtiter plate(s) & lids.
6. Remove confluent culture flask(s) from incubator & place under hood.
7. Wash gloves w/ 70% EtOH.
8. Remove cap(s) from flask(s) & decant media into waste beaker.
9. Using a sterile pipet wash cells 3 X w/ 10 mL of buffer (PBS for H4IIE & Cortland's for PLHC) decant into waste beaker.
10. Using a sterile pipet, add 4 mL Trypsin (0.01 uM/H4IIE, 0.005 uM w/ EDTA/PLHC) to flask(s) & allow time for cells to be released (incubate H4IIE cells 5-10 min. till cells float, PLHC 3-5 min. till cells float). Replace lid during incubation and tap flask to loosen cells!
11. Immediately stop trypsinization by pipetting (sterile pipet) 6 mL of D-MEM to flask(s) (15% F.B.S./H4IIE, 10% F.B.S./PLHC). DO NOT PROLONG TRYPSIN EXPOSURE!
12. Using a sterile pipet, pipet the 10 mL of Trypsin/D-MEM/cells solution into a sterile 50 mL centrifuge tube & wash flask 1to 3 times w/ D-MEM (15% F.B.S./H4IIE, 10% F.B.S./PLHC).
13. Cells are often clumped, so break apart by vortexing or pipetting vigorously.
14. Get cell concentration of solution w/ hemacytometer:
    a. Using a sterile tip, place 10 ul of stock solution on each side of hemacytometer counting grid under the cover slip (use new tip for each side).
    b. Under microscope count all cells in 4 of the 16 division areas above the center trough & 4 of the 16 division areas below the center trough, record counts.
    c. Average the 8 counts & multiply by 10^4, this = the # of cells/mL in the stock solution.
15. Calculate the number of cells needed for the day's seeding:
    a. Need 7000 cells/well (300 µL) or 23,000 cells/mL for H4IIE
       Need 20,000 cells/well (300 µL) or 67,000 cells/mL for PLHC
    b. Multiply # of cells/well needed by total # of wells to be seeded, this = the total # of cells needed for the day's seeding.
16. Calculate the volume of seeding stock solution (D-MEM & cells) required:
    a. Need 300 µL D-MEM in each well
    b. Multiply 0.3 ml by total # of wells to be seeded, this = the total volume (in mL) of seeding solution needed.
    c. Use the following formula to determine the dilution factor for the cell solution (obtained in step IV, A, 14 above).

$$\frac{\text{[cell suspension currently]}}{\text{[desired cell solution]}} = \text{dilution factor}$$

Example: If cells solution concentration is at 1 X 10^6 cells/mL & desired concentration is 23,000 cells/mL, then, 1,000,000/23,000 = 44. Therefore make the volume of seeding stock needed by taking 1 part cell suspension to 43 parts D-MEM.

17. Make seeding solution w/ cell stock solution & D-MEM in a sterile Erlenmeyer flask or 50 mL centrifuge tube.
18. Decant seeding solution into sterile reagent well.
19. Using the 1250 µL 8-channel pipettor, pipet 300 ul of seeding solution into each well to be seeded (for H4IIE EROD assay leave wells A5, A6, A7 blank).
20. Place lids on plates & label w/ date, cell type & your initials & place in incubator (37^o C, 5% CO_2 for H4IIE, 32^o C, 5% CO_2 for PLHC).
21. Put everything away & once again wipe working area of hood down w/ 70% EtOH.
22. Turn off hood.

B. Dosing plated cells:
Note: Use sterile techniques during this procedure. All standards and environmental samples must be dissolved in iso-octane and are dosed in 5 µL aliquots. Four replicates are dosed at each concentration.

1. TCDD standard. TCDD is dosed in concentrations ranging from 50 pg/well to 0.069 pg/well (Figure. 1). Dosing concentrations (10 pg/uL to approximately 0.014 pg/uL) are made from the TCDD stock of 10 pg/uL iso-octane (see attachment) by the following procedure:

   Four to six TCDD curves are run each assay day. These curves should be dosed in varying sections of six different plates with sample or basal dilutions on the rest of the plate. Dilutions from the stock solution are made via the following:

   a. Place 8 dosing vials into a holding rack.
   b. Calculate amount of stock needed for dilutions using the formula:

[5 µL X (# of reps)] X (# of curves) = # uL needed + 10 uL = A
The 10 µL is added to this figure to allow for residual in vials that cannot be removed by pipettor (this value can be adjusted to result in whole numbers for easier calculation).

A + 1/2(A) = total µL stock (B)

c. Place total amount of stock (**B*)*** calculated above into the highest concentration dosing vial (# 1).
d. Place **A** µL of iso-octane into each of the remaining dosing vials (# 2 thru # 8).
e. Make a 1:2 (3-fold) serial dilution of the concentrated stock of TCDD. Take **A**/3 µL of the high standard (from vial # 1) and place into vial # 2 & mix, this is the first dilution. Continue the dilution series for the next 6 vials. Stop at vial # 7 so that # 8 is an iso-octane blank.

f. Obtain a 20 µL 8-channel repeating pipettor set to deliver 5 µL four times and , using sterile tips, aspirate 20 µL of each dilution. Deliver 5 uL of each dose to each well starting at column 1, 5, or 9 as in Figure 1.

g. After dosing of standard is complete, pipet the remaining residual standard back into vials, then place all vials in an appropriate container so they can later be crushed, solvent rinsed and disposed. The liquid waste should be photolyzed for 24 hrs. Tips are then ejected into an appropriate waste container for later solvent rinse and disposal.

Figure 1

Dosing direction → (values reported in pg/well)

	1	2	3	4	5	6	7	8	9	10	11	12
A	no cells	no cells	no cells	iso cntrl	iso cntrl	iso cntrl	iso cntrl	iso cntrl	iso cntrl	iso cntrl	iso cntrl	iso cntrl
B	TCDD 0.069	TCDD 0.069	TCDD 0.069	TCDD 0.069								
C	TCDD 0.21	TCDD 0.21	TCDD 0.21	TCDD 0.21								
D	TCDD 0.62	TCDD 0.62	TCDD 0.62	TCDD 0.62								
E	TCDD 1.85	TCDD 1.85	TCDD 1.85	TCDD 1.85								
F	TCDD 5.6	TCDD 5.6	TCDD 5.6	TCDD 5.6								
G	TCDD 16.7	TCDD 16.7	TCDD 16.7	TCDD 16.7								
H	TCDD 50.0	TCDD 50.0	TCDD 50.0	TCDD 50.0								

2. Environmental Samples:
   Sample dilutions should be chosen to facilitate a complete Dose Response curve. These are made in the same procedure as that of the TCDD Standard. Usually the following format is used:
   4-fold dilution, repeated for 6 dilutions, iso-octane blank, and 4 replicates of each dilution (Figure. 2) using the following procedure:
   a. Place 8 dosing vials into a holding rack.
   b. Calculate amount of stock needed for dilutions using the formula:

      [5 ul (# of reps)] x (# of curves) = # uL needed + 10 µL = A
      The 10 µL is added to this figure to allow for residual in vials that cannot be removed by pipettor.

      A + 1/3(A)=total uL stock (B)

c. Place total amount of stock **(B)** calculated above into the first dosing vial
d. Place volume **A** of iso-octane into all remaining dosing vials.
e. Make a 1:3 (4-fold) serial dilution of the sample stock concentration. Take **A**/4 µL of the sample stock (from vial # 1) and place into vial # 2 & mix, this is the first dilution. Continue the dilution series for the next 6 vials. Stop at vial # 7 so that # 8 is an iso-octane blank.
f. Obtain a 20 µL 8-channel repeating pipettor set to deliver 5 µL four times and , using sterile tips, aspirate 20 µL of each dilution. Deliver 5 uL of each dose to each well starting at column 1, 5, or 9 as in Figure 2.
Approximately 10% (1 out 10) of the samples should be dosed in triplicate for each set of samples run.
g. After dosing of sample is complete pipet the residual solution back into vials, then place all vials in an appropriate container so they can later be solvent rinsed and disposed. The liquid waste should be photolyzed for 24 hrs. Tips are then ejected into an appropiate waste container for proper later disposal.

3. Allow plates to incubate for 72 hrs.

Figure 2

Dosing direction →

	1	2	3	4	5	6	7	8	9	10	11	12
A	Cells, no NADPH	Cells, no NADPH	Cells, no NADPH	iso cntrl	No cells	No cells	No cells	iso cntrl	iso cntrl	iso cntrl	iso cntrl	iso cntrl
B	S1,D6 R1	S1,D6 R2	S1,D6 R3	S1,D6 R4	S2,D6 R1	S2,D6 R2	S2,D6 R3	S2,D6 R4	S3,D6 R1	S3,D6 R2	S3,D6 R3	S3,D6 R4
C	S1,D5 R1	S1,D5 R2	S1,D5 R3	S1,D5 R4	S2,D5 R1	S2,D5 R2	S2,D5 R3	S2,D5 R4	S3,D5 R1	S3,D5 R2	S3,D5 R3	S3,D5 R4
D	S1,D4 R1	S1,D4 R2	S1,D4 R3	S1,D4 R4	S2,D4 R1	S2,D4 R2	S2,D4 R3	S2,D4 R4	S3,D4 R1	S3,D4 R2	S3,D4 R3	S3.D4 R4
E	S1,D3 R1	S1,D3 R2	S1,D3 R3	S1,D3 R4	S2,D3 R1	S2,D3 R2	S2,D3 R3	S2,D3 R4	S3,D3 R1	S3,D3 R2	S3,D3 R3	S3,D3 R4
F	S1,D2 R1	S1,D2 R2	S1,D2 R3	S1,D2 R4	S2,D2 R1	S2,D2 R2	S2,D2 R3	S2,D2 R4	S3,D2 R1	S3,D2 R2	S3,D2 R3	S3,D2 R4
G	S1,D1 R1	S1,D1 R2	S1,D1 R3	S1,D1 R4	S2,D1 R1	S2,D1 R2	S2,D1 R3	S2,D1 R4	S3,D1 R1	S3,D1 R2	S3,D1 R3	S3,D1 R4
H	S1,STK R1	S1,STK R2	S1,STK R3	S1,STK R4	S2,STK R1	S2,STK R2	S2,STK R3	S2,STK R4	S3,STK R1	S3,STK R2	S3,STK R3	S3,STK R4

S=sample, D=dilution, R=replicate, STK=stock

C. EROD assay

1. Take plates out of incubator and wash with ultra pure water in a 96-well plate washer set at 300 uL wash, 2 sec. soak time, 3 cycles (stage set to leave approx. 20 uL residual ultra pure water.)

2. Place plate in incubator for 5 min. to allow for cell lyses due to osmotic force.

3. Add 20 uL PBS buffer at (30°C - PLHC-1, 37°C H4IIE) with 80 uM Dicumarol to each well (final Dicumarol concentration in each well=20 uM). Then add an extra 20 µL to wells A1, A2, & A3.

4. Add 20 uL of 5 uM Ethoxyresorufin to all wells for a final concentration of 1.25 uM in each well.

5. Add 20 uL of 5mM NADPH solution to all wells for a final concentration of 1.25 mM in each well except wells A1, A2, & A3.

6. Place the microtiter plate into the Fluorometric plate reader (Cytofluor 4000) and scan at settings:

Plate Type:	Nunc 96 △
Reads/well:	3
Scans/cycle:	1
Cycle Time:	1 min
Cycles:	20 for samples, 10 for resorufin std.
Excitation Filter:	530 nm
Emission Filter:	580 nm
Gain:	55

7. After plate readings are taken choose export/print from the file menu, save the data in a noted file giving it an appropriate name (i.e. s for sample, the date, then a letter of the alphabet for each plate, **s012202a**) to an EXCEL spreadsheet as a linear .CSV file for data analysis.

8. At the end of the day run one plate for both Resorufin and protein standards.

9. Run Resorufin standards first.

   a. Take a 96 well microtiter plate and wash as in step 1 of EROD assay procedure.
   b. Add 20 uL of Resorufin standards (see appendix for preparation) to the first 6 columns of the plate according to the format in Figure 3.
   c. Repeat steps 4, 5, 6, and 7 of EROD assay procedure.

Figure 3

@ Resorufin stds. *BSA stds.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS Blnk	PBS Blnk	PBS Blnk	PBS Blnk	PBS Blnk	PBS Blnk	PBS Blnk	PBS Blnk	PBS Blnk	PBS Blnk	PBS Blnk	PBS Blnk
B	5	5	5	5	5	5	1.875	1.875	1.875	1.875	1.875	1.875
C	10	10	10	10	10	10	3.75	3.75	3.75	3.75	3.75	3.75
D	20	20	20	20	20	20	7.5	7.5	7.5	7.5	7.5	7.5
E	40	40	40	40	40	40	15	15	15	15	15	15
F	80	80	80	80	80	80	30	30	30	30	30	30
G	160	160	160	160	160	160	60	60	60	60	60	60
H	320	320	320	320	320	320	120	120	120	120	120	120

@ Amounts reported in total pmols resorufin/well
* Amounts reported in total ug BSA/well

D. Fluorescamine Protein Assay

1. Place BSA protein standards (1.56 - 25ug/well) in columns 7 -12 following the scheme as in fig. 3. (The standard plate is used as an external curve for all other plates).

2. Add 150 uL $NaPO_4$ buffer to standard plate then place in plate shaker for 1 minute at medium speed.

3. Add 20 ul of 1.08 mM/20 ul fluorescamine in acetone to each well and shake 1 minute at medium speed.

4. Allow reaction to take place for 5 to 15 minutes. Choose a reaction time and use that time for all protein analyses.

5. Place the microtiter plate into the Fluorometric plate reader (Cytofluor 4000) and scan at settings:

Plate Type:	Nunc 96 △
Reads/well:	3
Scans/cycle:	1
Cycle Time:	1 min

Cycles:	1
Excitation Filter:	400 nm
Emission Filter:	460 nm
Gain:	55

6. Repeat steps 3 - 6 for all other plates. Save, export, and label files as described in step 7 of Resorufin analysis described above.

V. Calculation of EROD activity

A. Protein Standard Analysis

1. Open protein standard .csv file. Run the **pstdanalysis** macro by pressing **ctrl p**.
2. Save the file as an excel file.
3. Check that the r^2 is 0.95 or higher.

B. Resorufin Standard Analysis

1. Open Resorufin standard .csv file. Run the **resstdanalysis** macro by pressing **ctrl r**.
2. Save the file as an excel file.
3. Check that the r^2 is 0.95 or higher.

C. Protein Sample Plate Analysis

1. Open sample protein .csv file. Go to the protein standard .xls file, select the slope and y-intercept, the top 2 cells of the linest array, and using the paste special, values, transpose function place the slope & y-intercept into cells G1 & G2, respectively, of the sample protein .csv file.
2. Run the **ERODbioassayproteincalc** macro by pressing **ctrl t**.
3. Save the file as an excel file.

D. EROD Sample Plate Analysis

1. Open sample .csv file. Insert a column at Column A.
2. Open the corresponding sample protein .xls file, copy the mg protein values and using the paste special, values function place these values in the inserted column A starting at cell A31.
3. Go to the resorufin standard .xls file, select the slope and y-intercept, the top 2 cells of the linest array, and using the paste special, values, transpose function place the slope & y-intercept into cells G1 & G2, respectively, of the sample .csv file.
4. Run the **ERODbioassayanalyze** macro by pressing **ctrl z**.

5. Save as an excel file.
6. Go through the data by graphing the pmols of resorufin produced over the 20 minutes and use that graph and the highest pearson correlation to change the linest calculation so that the slope used to calculate the EROD rate is that of the linear part of the curve. This must be done for every well.
7. Run the **BioassayERODdatacollect20** macro by selecting the tools, macro, and macro run buttons. The data is now ready to be used in the dose response calculation.

E. EROD Dose Response Calculation

1. Open the DoseResponse A1.xls and the DoseResponse B1.xls files and copy the protein values and EROD rate values into the appropriate columns.
2. Place the sample names in the appropriate cells.
3. Place the number of grams of tissue extracted into the appropriate cells.
4. Go through the columns of data and mark (by coloring the cells) those data points that have obvious aberrations (i.e. those with very low protein values, negative EROD rate values, those that vary dramatically from the others in their dose range, etc.).
5. Run the **ERODbioassydrcopysort** macro and then delete those points in the copied area that were marked in the previous step. Make sure that there are no blank cells among the data points.
6. Run the **ERODbioassaydrpearsoncalc** macro, then the **ERODbioassaypearsoncopysort** macro split the screen and then run the **ERODbioassaydrlinest** macro.
7. Using those cells with the highest positive pearson correlation indicated by the sorted pearson data, change the linest array for that data so that the most positive linear portion of the dose response curve is used to calculate the slope.
8. Run the **ERODbioassaydrslopecollect** macro.
9. Repeat steps 2 through 8 for each page on the DoseResponse A1.xls and the DoseResponse B1.xls files.
10. Copy the Sample ID, Slope, se, and r^2 below the compiled data on the abcd sheet of the DoseResponse A1.xls file. Then, copy the compiled data from each of the other three sheets to this sheet into the same table. Use the Data, sort command to sort the data points.

F. Calculation of LOD & LOQ

1. Insert a sheet into the DoseResponse A1.xls file. Label this sheet LOD & LOQ. Copy all Basal artificial doses and EROD rates to this sheet in two labeled columns.

2. Use the Tukey Outlier Rule to detect outliers in each set of basal EROD rates. Remove any outliers. Because all of these EROD rates were measured on wells to which nothing was added they can be considered the same.
3. Using the Linest function calculate the statistic array including the slope, standard deviation, r^2, etc.
4. Use the calculated slope and standard deviation to calculate LOD and LOQ using the following equations.
   LOD = EROD rate + 3(SD)
   LOQ = EROD rate + 10(SD)

G. Average TCDD standard data

1. On the DoseResponse A1.xls file, calculate the average Slope, SD & r^2 for the six TCDD curves.
2. Use these averages in the TCDD-EQ Table.

H. TCDD-EQ Calculation and Data Summation

1. Open the TCDD-EQ Template file and copy data to the appropriate columns
2. Place LOD and LOQ values in the appropriate cells.
3. Fill in the other appropriate data.

I. Explanation of Macros

1. **pstdanalysis:**

   a. Calculates the average AFU for all six wells at each concentration.
   b. Makes a table with a column for nominal BSA concentration, a column for Average AFU at each concentration, and a column for Average AFU with the background subtracted (so that the blank was 0).
   c. Places the nominal BSA concentrations in the appropriate column. Copies the average AFU's for each concentration into the appropriate column. Then subtracts the blank average AFU from each of the other averages and places the answer into the appropriate column.
   d. Calculates a straight line that best fits the Average AFU with background subtracted and the nominal resorufin concentration data using the Excel LINEST function. The command is typed into a 5-cell by 5-cell array so that it returns the following set of statistics describing that line.

m_n	(slope)	m_b	(slope intercept)
se_n	(std. Error of slope)	se_b	(std. Error of intercept)
r^2	(coefficient of determination)	se_y	(std. Error for y)
F	(F-observed value)	df	(degrees of freedom)
ss_{reg}	(regression sum of squares)	ss_{resid}	(residual sum of squares)

Note that Excel uses the "least squares" method to make these calculations.

e. The slope and the slope intercept are then ready to be copied into the sample files for analysis on each plate.

2. **resstdanalysis:**

a. Calculates the average AFU for all six wells, 10 scans at each concentration.

b. Makes a table with a column for nominal resorufin concentration, a column for Average AFU at each concentration, and a column for Average AFU with the background subtracted (so that the blank was 0).

c. Places the nominal resorufin concentrations in the appropriate column. Copies the average AFU's for each concentration into the appropriate column. Then subtracts the blank average AFU from each of the other averages and places the answer into the appropriate column.

d. Calculates a straight line that best fits the Average AFU with background subtracted and the nominal resorufin concentration data using the Excel LINEST function. The command is typed into a 5-cell by 5-cell array so that it returns the following set of statistics describing that line.

m_n	(slope)	m_b	(slope intercept)
se_n	(std. Error of slope)	se_b	(std. Error of intercept)
r^2	(coefficient of determination)	se_y	(std. Error for y)
F	(F-observed value)	df	(degrees of freedom)
ss_{reg}	(regression sum of squares)	ss_{resid}	(residual sum of squares)

Note that Excel uses the "least squares" method to make these calculations.

e. The slope and the slope intercept are then ready to be copied into the sample files for analysis on each plate.

Attachments to SOP C5.194

Phosphate Buffered Saline, PBS (pH 7.8)

Component	Quantity
Sodium Chloride (NaCl) Sigma S9625	8.0 g
Potassium Chloride (KCl) Sigma P4504	0.2 g
Sodium Phosphate Dibasic (Na_2HPO_4) Sigma S0876	1.15 g

Sodium Phosphate Monobasic (NaH_2PO_4) Sigma S0751	0.2 g
Ultra pure Water	1000 mL

1. Put approximately 800 mL of the U.P. water in a 1 L graduated cylinder and place on a stir plate w/ stir bar in motion.
2. Add all components dissolving first in the weigh boat and then pouring into the stirring U.P. water.
3. Bring up to volume with U.P. water.
4. Titrate pH to 7.8 with 5N NaOH or 6N HCl.
5. Decant into clean, 500 mL glass bottles.
6. Store with cap tightly secured at ~ 4^o C.

1.08 µM Fluorescamine

Component	Quantity
Fluorescamine Sigma F9015	0.039 g
Acetone	52 mL

1. Mass fluorescamine on acetone rinsed Aluminum foil or in an acetone rinsed foil weigh boat.
2. Place ~ 45 mL acetone in a glass graduated cylinder; add the fluorescamine rinsing with acetone into the graduated cylinder.
3. Bring up to 52 mL with acetone.
4. Decant into a clean, brown or foil covered glass bottle fitted with a silicone lined cap. The acetone will evaporate readily if the cap is not secured well.
5. Store at ~ 25^o C in a dark area.

Ethoxyresorufin Solutions

200 µM Super Stock

Dissolve 5 mg of ethoxyresorufin (Molecular Probes R-352) in 103.7 mL of methanol. Store in a clean dark glass or foil covered bottle and seal with Para film at ~ -8^o C. This solution is very light sensitive and should be labeled as such and exposed to light as little as possible.

5 µM solution

Add 1 mL of the 200 µM super stock to 39 mL of PBS.
Measure the concentration of this solution as described in section VI. D. of this document.

5 mM β-NADPH Solution

1. Mass 0.083 g of β-NADPH (ICN 101167) in a small weigh boat.
2. Dissolve in weigh boat with PBS.
3. Pipet solution into a 50 mL conical centrifuge tube.
4. Rinse weigh boat with PBS into centrifuge tube and bring up to 20 mL.
5. Store covered at ~ -8° C. Light sensitive and best if used the same day made.
6. Measure the concentration of this solution as described in section VI. D. of this document.

Resorufin Solutions

850 µM Super Stock

1. Mass 5 mg of resorufin (Molecular Probes R-363) into a small weigh boat.
2. Dissolve in weigh boat with methanol.
3. Pipet solution into a 50 mL conical centrifuge tube.
4. Rinse weigh boat with methanol into centrifuge tube and bring up to 25 mL.
5. Cover the centrifuge tube with aluminum foil.
6. Store covered and sealed w/ Para film at ~ -8° C. This solution is very light sensitive and should be labeled as such and exposed to light as little as possible.

16 µM Stock (16 pmol/µl or 320 pmol/20 µl) For Resorufin Standard

Add 480 µl of the 200 µM resorufin super stock to 5520 µl (5.52 mL) PBS in a 15 mL centrifuge tube.
Measure the concentration of this solution as described in section VI. D. of this document. Use the same day it is made.

Resorufin Standards

1. Place 1 mL of the 16 µM resorufin (320 pmols/20 µl) in a 1.2 mL cryovial.
2. Place 500 µl of PBS in each of seven 1.2 mL cryovials.
3. Make a 1:1 series dilution of the 16 µM resorufin for 6 dilutions. Leaving the last as a blank. The resulting concentrations will be as follows:

320 pmols/20 µl
160 pmols/20 µl
80 pmols/20 µl
40 pmols/20 µl
20 pmols/20 µl
10 pmols/20 µl
5 pmols/20 µl
0 pmols/20 µl

Protein (BSA) Standards

1. Make new Daily. Mass 24 mg of bovine serum albumin (BSA), Sigma A2153, in a small weigh boat.
2. Dissolve in weigh boat with 4 mL of PBS.
3. Pipet 1 mL of the solution into 1 mL micro tube (120 ug/20 µl), discard the rest.
4. Make the following dilutions:
   500 µl of the 120µg/20 µl into 500µl PBS = 60 µg/20 µl
   500 µl of the 60µg/20 µl into 500µl PBS = 30 µg/20 µl
   500 µl of the 30µg/20 µl into 500µl PBS = 15 µg/20 µl
   500 µl of the 15µg/20 µl into 500µl PBS = 7.5 µg/20 µl
   500 µl of the 7.5µg/20 µl into 500µl PBS = 3.75 µg/20 µl
   500 µl of the 3.75µg/20 µl into 500µl PBS = 1.875 µg/20 µl
   500 µl PBS as the blank.

Date Prepared: 1/16/91

Date Revised: 6/18/98

TISSUE ANALYSIS FOR PCBs AND LOW-LEVEL PLANAR HALOGENATED HYDROCARBONS

I. Introduction

This SOP describes a preparative scheme for tissue samples in which PCBs, PCDDs, and PCDFs are targeted. The purified extracts generated at the end of the procedure are ready for high performance gel permeation chromatography (HPGPC) followed by GC/ECD analyses (or GC/ECD semiquantitative screening), or fractionation by high performance carbon chromatography into coplanar fractions and PCDD-PCDF fractions. The extracts may be analyzed at ppt levels and therefore must be of sufficient quality to be reduced to a final volume of as low as 10 μL. The sequence of steps described in this SOP includes blending of the tissue with sodium sulfate, extraction, percent lipid determination, and reactive cleanup. HPGPC is detailed in SOP P.464.

II. Safety Considerations

In performing the operations delineated in this SOP, all aspects of the CERC Safety Plan shall be adhered to. Volatile or flammable solvents shall be handled only in the fume hood. Personnel shall wear lab coats, gloves, safety glasses and other appropriate protective gear. Radiolabeled materials such as ^{14}C-2,3,7,8-TCDD shall be worked with only above the specially designed absorbent paper. This is only a partial listing of relevant safety precautions. Analysts should refamiliarize themselves with the Safety Plan before embarking on this project.

III. Listing of Reagents and Apparatus

A. Glassware - *before use, rinse <u>all</u> glassware with acetone, hexane, toluene, and dichloromethane.*

B. Anhydrous sodium sulfate (Na_2SO_4) prepared as per CERC SOP C5.8

C. Silica gel SG-60 prepared as in CERC SOP P.270.

D. Standard 40/60; W/W; sulfuric acid/silica gel (SA/SG) prepared as per CERC SOP P.270.

E. Coarse 30/70; W/W; sulfuric acid/silica gel (COARSE SA/SG) prepared in accordance with CERC SOP P.270.

F. Standard potassium silicate (KS) prepared as per CERC SOP P.271.

G. Blender blades, stainless steel rods and spatulas. Preparation: Rinse this equipment with acetone, hexane, then dichloromethane.

IV. Blending Tissue with Sodium Sulfate

This SOP assumes that you will be starting with ground tissue samples. The day before you plan to extract a group of tissue samples, thaw the samples and proceed as follows: Weigh aliquants (usually 15-g wet weight portions) of the tissue samples into half-pint canning jars. Add four times the sample wet weights of Na_2SO_4 to the jars. Immediately and thoroughly stir the tissue-sodium sulfate mixture using stainless steel rods or spatulas. Allow the mixture to react for about an hour before stirring again. Occasionally stir the samples throughout the day to prevent hardening. The next morning, thoroughly stir the mixtures one more time. Homogenize the mixture with a blender until it has a powdery consistency.

V. Extraction

NOTE: Due to the tremendous concentration factors involved in this procedure (15g. sample to a few microliters), it is essential that special attention be given to cleanliness and proper technique. All sample contact surfaces (columns, flasks, test tubes, caps, etc.) should be cleaned immediately before use by generous rinsing with acetone, hexane, toluene, and dichloromethane.

In the past, the current method of column extraction was compared to soxhlet and shake extraction, using positive control sediment (Saginaw Bay sediment) as the control matrix. It was found that, while soxhlet did average a higher percentage of recovery of most of the contaminants measured, column extraction averaged approximately 90% of the recoveries of soxhlet. It was decided that the slightly higher extraction efficiency of soxhlet did not warrent the substantial increase in cost, labor, time and complexity. (Shake extraction was the least efficient, averaging about 65% of the efficiency of soxhlet.)

A. Prepare 2-cm i.d. extraction columns as follows. Insert stopcocks in the columns and rinse with acetone, hexane, toluene, and dichloromethane. When the columns have dried, place wads of glass wool at the bottoms of the columns and cover the wads completely with sodium sulfate. Rinse the insides of the column assemblies with a few mL of dichloromethane. Pour in the freshly blended tissue-sodium sulfate mixture and tap the columns to settle the mixture, eliminate voids, and level the top surface of the mix.

B. Spike surrogates (procedural internal standards) and, when applicable, radiolabeled and cold analytes into the tissue-Na_2SO_4 mixtures at this point.

Using an appropriately sized syringe, dispense the correct volume of spiking solution onto the sample mix. Rinse any remaining spiking solution from the syringe needle tip onto the matrix with a small volume of methylene chloride dispensed from a pasteur pipet.

C. After spiking, deposit Na_2SO_4 to segment heights of about 2 cm over the packed material, pouring the sodium sulfate down the sides of the columns to dislodge the small particles of sample mix adhering to the sides. For all samples that received spikes, rinse the sides of the columns with 2-3 mL dichloromethane and allow them to stand for 30 minutes before proceeding further. Place 500-mL boiling flasks that have been rinsed with the same sequence of solvents as the columns beneath the columns. Open the stopcocks, and carefully pour 30 mL of dichloromethane into each solvent reservoir. When the levels of the solvent in the reservoirs have descended to approx. 1 cm. above the tops of the upper Na_2SO_4 segments, close the stopcocks and allow the tissue-desiccant mixtures to soak in the CH_2Cl_2 for at least an hour. Then, allow the extraction solvent level to decend to the top of the sodium sulfate layer and gently pour 150 mL of CH_2Cl_2 into the reservoirs and open the stopcocks to obtain $\leq$2 drops/second flow. When this volume of solvent has drained from the column completely, add another 30 mL methylene chloride to the columns and collect it. Keep the reservoirs covered with aluminum foil except when adding solvent.

VI. Determination of Percent Lipid

Rotary evaporate the tissue extracts to volumes of ~10 - 20 mL. Use CH_2Cl_2 rinses to transfer the lipid solutions to 50-mL volumetric flasks and to dilute them to the 50-mL marks. For details of lipid determination, the analyst is referred to CERC SOP P.461.

VII. Reactive Adsorbent Cleanup - First Stage

The following preliminary cleanup step can accommodate no more than **one gram** of total lipid per chromatography column. If you have verified (in Step VI.) that any of the volumetric flasks contain more than one gram of lipid, split the extracts in two or more approximately equal portions for the chromatographic/reactive cleanup described below. Each portion of a split sample will then be applied to its own chromatography column, and the eluates recombined afterward.

A. Prepare 2-cm i.d. glass chromatography columns by attaching stopcocks and then placing wads of glass wool in the bottoms of the columns. Deposit anhydrous sodium sulfate over the glass wool to segment heights of ~1 cm.

B. For the sake of convenience and consistency, the adsorbents will be dispensed by volume using a graduated cylinder. (When measuring the volumes, tap or shake the cylinder in a consistent manner to settle the material being

measured, thereby acheiving an accurate measurement. It is not necessary to carry this "settling" operation to extremes). First, add 10 mL of KS to each column. Tap each column to settle the adsorbent and produce a level top surface. Next, add 10 mL SA/SG, again settling and leveling. Place a 1-cm layer of sodium sulfate over the SA/SG. Finally, add 15 mL COARSE SA/SG to the columns and settle. Wash the columns with 25-30 mL of dichloromethane, allowing the solvent to descend just to the top of the adsorbent (discard this solvent).

C. Quantitatively apply the remaining (after % lipid determinations) lipid solutions to the columns described above. Open the stopcocks and allow the samples to sink into the adsorbent. Observe the discoloration resulting from the sample's reaction with the COARSE SA/SG and close the stopcock when the discoloration has reached the sodium sulfate layer that separates the two acidic adsorbents. Stir this COARSE SA/SG layer thoroughly with a metal spatula to remove the gas bubbles that will have evolved there. Disturb the sodium sulfate layer as little as possible. The stirring process will cause discoloration of the remaining sample solution that has not passed into the adsorbent. Rinse the residue on the spatula back into the columns thoroughly with dichloromethane. Open the stopcocks and, when the sample solutions have descended into the adsorbent, rinse each volumetric flask with three 5-mL portions of CH_2Cl_2, adding these rinsings to the column. When these rinses have descended into the adsorbent, wash the column walls with another three 3-mL portions of dichloromethane, allowing each one to sequentially sink into the column. Then close the stopcock and deposit approximately 1 cm of sodium sulfate onto the top of the column. Gently pour 60 mL of CH_2Cl_2 into the column reservoirs. Open the stopcocks to obtain a flow rate of approximately 2-3 drops/second and collect all effluent in 125-mL boiling flasks.

NOTE: In some instances, the total lipid content of individual samples within a set may consistently fall above 1 gram. For samples which contain >1 gram but ≤4 grams lipid, the reactive column configuration may be altered. A 4-cm i.d. is used in place of the 2-cm one. The volumes of the adsorbents are adjusted to 25 mL KS, 25 mL SA/SG, and 50 mL COARSE SA/SG. The column is presaturated with 80-100 mL dichloromethane. The column is eluted with 150 mL dichloromethane, collected at 2-3 drop/second into a 500 mL flask.

VIII. Reactive Adsorbent Cleanup - Second Stage

A. Rotary evaporate extracts to volumes of about 20 mL, add 6 mL of isooctane to each flask, and rotary evaporate to 3-mL volumes. Use identical flasks containing 20 and 3 mL of solvent to make volume comparisons if necessary.

B. Clean extracts up on silica gel, KS, and SA/SG as follows. In 1.0-cm i.d. columns, pack the following ingredients from bottom to top: a plug of glass wool; a 1-cm segment of anhydrous sodium sulfate; a 10-mL segment of silica gel SG-60: a 3-mL segment of KS; a 5-mL segment of standard 40/60 SA/SG; and a 1-cm segment of anhydrous sodium sulfate. Presaturate the column with the eluant, 3% dichloromethane; 97% hexane (V+V). Place 125-mL flat bottom flasks underneath the columns. Transfer the extracts to the tops of the adsorbent columns and rinse the 500-mL flasks with three 3-mL portions of the eluant, sequentially applying the rinsings to the columns. Gently pour 45 ml of the eluant into the reservoirs and allow it to pass into the flasks at 1 drop/second (3 mL/min.) flow rate.

C. Add 1-2 mL of isooctane to each flask and rotary evaporate extracts to about 3 mL. Transfer the extracts to graduated culture tubes through pasteur pipet-filters. These filters are prepared by tamping small wads of 3-micron GFD filter into the bottoms of pasteur pipets and passing a 2-3 ml methylene chloride rinse through the assemblies to remove any extraneous material or stray glass fibers. Place the filters in the receiving culture tubes and transfer the concentrated extracts through them. Rinse the sample flasks with three sequential methylene chloride rinses, passing them through the filters also. The sample extracts may now be reduced in volume and transferred to autosampler vials in preparation for HPGPC (CERC SOP P.464). After HPGPC, the samples can proceed to fractionation on HPLC-Carbon or to GC/ECD analysis/screening after the appropriate solvent exchange.

Prepared by:

John Meadows
Research Chemist

Approved by:

_____
Section Leader, Organic Chemistry

_____
Quality Assurance Officer